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ANDIARA ESPÍNDOLA DE FREITAS

Mecanismos envolvidos na ação antidepressiva de *Tabebuia avellanedae*

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Orientadora: Prof[a]. Ana Lúcia Severo Rodrigues

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"Mecanismos envolvidos na ação antidepressiva de *Tabebeuia avellanedae*"

por

Andiara Espíndola de Freitas

Dissertação julgada e aprovada em sua forma final pelos membros titulares da Banca Examinadora (Port. 07/PPGBQA/2012) do Programa de Pós-Graduação em Bioquímica - UFSC, composta pelos Professores Doutores:

Banca Examinadora:

Ana Lúcia Severo Rodrigues

Prof(a) Dr(a) Ana lúcia Severo Rodrigues
(presidente/BQA/CCB/UFSC)

Prof(a) Dr(a) Manuella Pinto Kaster (CCVS/UCPel)

Adair Roberto Soares dos Santos

Prof(a) Dr(a) Adair Roberto Soares dos Santos **(CFS/CCB/UFSC)**

Prof(a) Dr(a) Prof(a) Dr(a) Marcelo Farina **(BQA/CCB/UFSC)**

Prof. Dr. Marcelo Farina
Coordenador do Programa de Pós-Graduação em Bioquímica

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"A inteligência é rica em méritos para o futuro, mas com a condição de ser bem empregada. Se todos os homens bem dotados se servissem dela segundo os desígnios de Deus, a tarefa dos Espíritos seria fácil, ao fazerem progredir a humanidade"

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LISTA DE ABREVIATURAS

AMP - Adenosina 5'-monofosfato
AMPc - adenosina 3',5'-monofosfato cíclico
ANOVA - análise de variância
ATP - adenosina 5'- trifosfato
BDNF - fator neurotrófico derivado do cérebro
BO - bulbectomia olfatória
CaMK - proteína quinase dependente de cálcio e calmodulina
CE - eletroforese capilar
CRE - elemento de resposta ao AMPc
CREB - proteína de ligação responsiva ao AMP cíclico
DO - densidade óptica
ERK1/2 - cinases 1 e 2 reguladas por sinal extracelular
FDA - administração de drogas e alimentos dos Estados Unidos
GCs - guanilato ciclase solúvel
GMPc - guanosina 5'-monofosfato cíclico
GMP- guanosina 5'-monofosfato
GSK-3β - glicogênio sintase cinase-3β
GTP - guanosina 5'-trifosfato
HPLC - cromatografia liquida de alta performance
5-HT - serotonina
5-HTT - transportadores de serotonina
iMAO - inibidor da monoamina oxidase
ISRS - inibidor seletivo da recaptação de serotonina
ISRSN - inibidor misto da recaptação de serotonina e noradrenalina
i.c.v. - intracerebroventricular
i.p. - intraperitoneal
MAO - monoamino oxidase
MAPK - proteína cinase ativada por mitógeno
NA - noradrenalina
NMDA - N-metil-D-aspartato
NO - óxido nítrico
NOS - óxido nítrico sintase
ODQ - 1H-[1,2,4]Oxadiazol[4,3-a]quinoxalin-1-ona]
PDE - fosfodiesterase
PI-3K - fosfatidilinositol 3'-cinase
PKA - proteína quinase A
PKB - proteína quinase B
PKC - proteína quinase C
PKG - proteína quinase dependente de GMPc
p.o.- *per oral* (via oral)
PVDF - polivinilideno de fluoreto
s.c. - subcutânea
SNC - sistema nervoso central
T. avellanedae - *Tabebuia avellanedae*
TCA - teste do campo aberto
TLC - cromatografia de camada delgada
TNF - teste do nado forçado
TNF-α - fator de necrose tumoral
TrkB - tropomiosina cinase B
TSC - teste da suspensão pela cauda

RESUMO

A bubectomia olfatória (BO) é um modelo animal de depressão bem estabelecido na literatura capaz de avaliar atividade antidepressiva após tratamento crônico e resulta em alterações comportamentais e bioquímicas que mimetizam vários sintomas de depressão. *Tabebuia avellanedae* Lorentz ex Griseb é uma planta utilizada na medicina popular da América tropical para o tratamento de sintomas depressivos. No presente estudo foi investigada a habilidade do tratamento repetido (14 dias) via oral (p.o.) do extrato de *T. avellanedae* de: a) produzir um efeito do tipo-antidepressivo no teste da suspensão da cauda (TSC); b) alterar fosforilação de Akt, GSK-3β, ERK1/2, CREB e o imunoconteúdo de BDNF hipocampal; c) reverter alterações comportamentais (hiperatividade e comportamento anedônico), bioquímicas (Akt, GSK-3β, ERK1/2, CREB e imunoconteúdo de BDNF hipocampal) induzidas pela BO em camundongos. A administração de extrato (10-30 mg/kg) durante 14 dias produziu um efeito do tipo-antidepressivo significativo no TSC. Adicionalmente, o extrato aumentou significativamente os níveis de fosforilação de CREB (Ser^{133}) e GSK-3β (Ser^{9}) (nas doses de 10-30 e 30 mg/kg, respectivamente). A BO causou um aumento de pCREB e pERK1 e do imunoconteúdo de BDNF. O tratamento com extrato preveniu a hiperatividade, anedonia, aumento de pERK1 e do imunoconteúdo de BDNF, de maneira similar à fluoxetina. Fluoxetina preveniu o aumento de pCREB induzido pela BO. Além disso, a fluoxetina causou uma diminuição significativa de pERK2 no grupo BO. Akt (Ser^{473}) não foi alterada em nenhum grupo experimental. Adicionalmente, o presente estudo se propôs a avaliar o envolvimento dos receptores NMDA e da via da L-arginina-NO-GMPc na ação do tipo-antidepressiva do extrato de *T. avellanedae* no TSC. O efeito anti-imobilidade do extrato (30 mg/kg, p.o.) foi prevenido pelo pré-tratamento dos camundongos com NMDA (0,1 pmol/sítio, i.c.v.), L-arginina (750 mg/kg, i.p., um substrato para a enzima óxido nítrico sintase) e sildenafil (5 mg/kg, i.p., um inibidor da enzima fosfodiesterase 5). Adicionalmente, a combinação de MK-801 (0,01 mg/kg, p.o., um antagonista não competitivo de receptor NMDA), 7-nitroindazol (25 mg/kg, i.p., um inibidor da NO sintase neuronal) e 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-ona (ODQ) (30 pmol/sítio i.c.v., um inibidor da guanilato ciclase solúvel) com uma dose sub-ativa de extrato (1 mg/kg, p.o.) produziu um efeito do tipo-antidepressivo sinérgico no TSC, sem causar nenhuma alteração significante na atividade locomotora dos animais. Em conjunto, os resultados mostram que o efeito tipo-antidepressivo do extrato pode estar associado com a fosforilação de CREB e GSK-3β. Além disso, a habilidade do extrato de prevenir as alterações comportamentais induzidas pela BO parece ser mediada pela via de neuroproteção da ERK1 e BDNF. Adicionalmente, os resultados sugerem que o efeito tipo-antidepressivo do extrato no TSC é dependente do bloqueio da ativação dos receptores NMDA e da inibição da síntese de NO-GMPc, extendendo dados da literatuta sobre a ação do tipo-antidepressiva desta planta, e ainda, reforçando o papel destes alvos para o mecanismo de ação de agentes antidepressivos. Nosso conjunto de resultados indica que esta planta pode se constituir uma estratégia atrativa para o manejo de sintomas de depressão agitada e/ou associada com anedonia.

ABSTRACT

The olfactory bulbectomy (OB) is a well-established animal model of depression capable of detecting antidepressant activity following chronic treatment and results in behavioral and biochemical changes reminiscent of various symptoms in depression. *Tabebuia avellanedae* Lorentz ex Griseb is a plant employed in tropical America folk medicine for the treatment of depressive symptoms. In this work we investigated the ability of the repeated (14 days) p.o. administration of the extract from *T. avellanedae* to: a) cause an antidepressant-like effect in the tail suspension test (TST); b) alter hippocampal Akt, GSK-3β, ERK1/2, CREB phosphorylation and BDNF immunocontent; c) reverse behavioral (hyperactivity and anhedonic behavior) and biochemical (hippocampal Akt, GSK-3β, ERK1/2, CREB, phosphorylation and BDNF immunocontent) changes induced by olfactory bulbectomy (OB), a model of depression, in mice. Extract (10-30 mg/kg) administered for 14 days produced a significant antidepressant-like effect in the TST. In addition, the extract increased both CREB (Ser^{133}) and GSK-3β (Ser^{9}) phosphorylation (at dose of 10-30 and 30 mg/kg, respectively). OB caused an increase in CREB and ERK1 phosphorylation and BDNF immunocontent. The extract prevented the OB-induced hyperactivity, anhedonia (loss of motivational and self care behavior), increased ERK1 phosphorylation and BDNF immunocontent, similarly to fluoxetine. Fluoxetine prevented the OB-induced increase in CREB phosphorylation. Moreover, fluoxetine caused a significant decrease in ERK2 phosphorylation in OB-group. Akt(Ser^{473}) was not altered in any group. In addition, this study was aimed to investigate the contribution of NMDA receptors and L-arginine-NO-cGMP pathway to the antidepressant-like action of the extract from *T. avellanedae* in the TST. The anti-immobility effect of the extract (30 mg/kg, p.o.) was prevented by pre-treatment of mice with NMDA (0.1 pmol/site, i.c.v.), L-arginine (750 mg/kg, i.p., a substrate for nitric oxide synthase) and sildenafil (5 mg/kg, i.p., a phosphodiesterase 5 inhibitor). In addition, the combination of MK-801 (0.01 mg/kg, p.o., a noncompetitive NMDA-receptor antagonist), 7-nitroindazole (25 mg/kg, i.p., a neuronal NO synthase inhibitor) and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (30 pmol/site i.c.v., a soluble guanylate cyclase inhibitor) with a sub-effective dose of extract (1 mg/kg, p.o.) produced a synergistic antidepressant-like effect in the TST, without causing significant alterations in locomotor activity. In conclusion, the antidepressant-like effect elicited by the repeated administration of the extract may be associated with CREB and GSK-3β phosphorylation. Additionally, the ability of the extract to prevent the behavioral changes induced by OB appears to be mediated by ERK1 and BDNF neuroprotection pathways. Furthermore, the results suggest that the antidepressant-like effect of the extract from *T. avellanedae* in the TST is dependent on a blockade of NMDA receptor activation and inhibition of NO-cGMP synthesis, significantly extending literature data about the antidepressant-like action of this plant and further reinforcing the pivotal role of these targets in the mechanism of action of antidepressant agents. Noteworthy, our results indicate that this plant could constitutive an attractive strategy for the management of agitated depression and/or depressive disorder associated with anhedonia.

1. Introdução

1.1. Depressão

A depressão é um transtorno mental crônico, recorrente, potencialmente fatal, com altas taxas de suicídio, intenso uso de tratamento e altos custos para sociedade (Nestler et al., 2002; Holtzheimer e Numeroff, 2006). Afeta 17% da população em todo o mundo (Kessler et al., 2005) e estima-se que um entre seis indivíduos norte-americanos será vítima desta doença no decorrer de sua vida (Kessler et al., 2005). Segundo projeções da Organização Mundial de Saúde, a depressão será a segunda principal causa de incapacidade nos países desenvolvidos até 2020 (Murray et al., 1997). Adicionalmente, além do elevado índice de mortalidade associado ao suicídio, pacientes deprimidos são mais propensos a desenvolverem doença arterial coronariana e diabetes mellitus tipo 2 (Van der Feltz-Cornelis et al., 2010). Sintomas depressivos incluem alterações somáticas e cognitivas, tais como: humor deprimido, anedonia (perda de interesse ou prazer em quase todas as atividades), irritabilidade, sentimentos de desvalia ou culpa, diminuição da capacidade de concentração, diminuição ou aumento do apetite, perda ou ganho de peso, insônia ou hipersonia, retardo ou agitação psicomotora, fadiga ou perda de energia, pensamentos recorrentes de morte e suicídio. Para o diagnóstico de depressão maior é necessária a constatação de no mínimo cinco entre estes nove sintomas, e exige a presença de pelo menos um dos dois primeiros sintomas mencionados (humor deprimido ou anedonia) presentes na maior parte do tempo, com uma duração mínima de duas semanas (Associação Americana de Psiquiatria, 2000).

A primeira hipótese formulada para explicar a etiologia da depressão postula que nesta doença há uma redução nos níveis sinápticos de noradrenalina (NA) e serotonina

(5-HT). A teoria monoaminérgica clássica (**Figura 1**) foi baseada no efeito antidepressivo de inibidores da enzima monoamina oxidase (MAO), de inibidores da recaptação de monoaminas e ainda no efeito depressivo da reserpina, fármaco que depleta as reservas monoaminérgicos nos neurônios (Schildkraut, 1965).

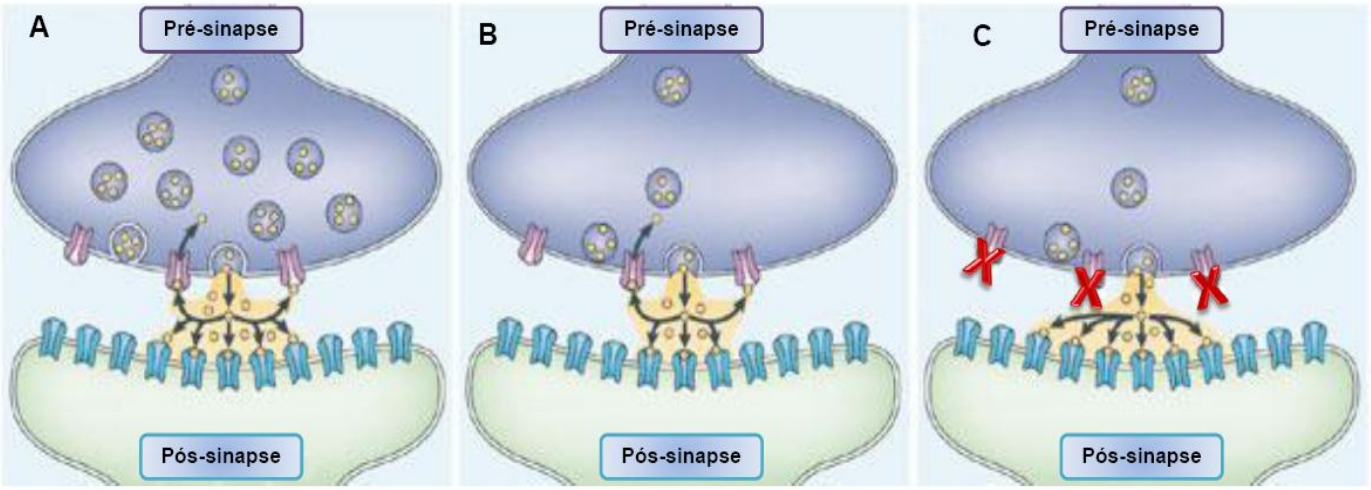

Figura 1. Teoria monoaminérgica da depressão. A. No cérebro normal os neurotransmissores monoaminérgicos (em amarelo) são liberados e ativam receptores pós-sinápticos. A neurotransmissão é terminada pela recaptação do neurotransmissor no neurônio pré-sináptico. **B.** Na depressão, a concentração de monoaminas na fenda sináptica está diminuída, acarretando o transtorno de humor**. C.** O bloqueio dos sítios de recaptação (em vermelho) aumenta a disponinibilidade dos neurotransmissores monoaminérgicos para ativarem seus receptores pós-sinápticos, e o humor é restabelecido. (Adaptado de Castrén, 2005).

Apesar de bem estabelecida, a hipótese monoaminérgica possui falhas: (i) não explica o fato de que os efeitos clínicos dos antidepressivos são observados apenas após cerca de 3-4 semanas do início da terapia, ao passo que estes medicamentos aumentam os níveis sinápticos de monoaminas de maneira muito mais eficiente (Elhwuegi, 2004); (ii) o fato de que a depressão está fortemente associada a fatores genéticos (Hyde et al., 2008) e a exposição a agentes estressores (Touma, 2011), e que apesar das inúmeras tentativas de associação entre variabilidade em genes relacionados a funções monoaminérgicas e situações de estresse em transtornos afetivos, uma relação

consistente ainda não está bem estabelecida (Harro e Oreland, 2001); (iii) nem toda droga que aumenta os níveis sinápticos de monoaminas tem ação antidepressiva (Baldessarini, 1996). Neste sentido, acredita-se que outros sistemas neurais e mecanismos bioquímicos estejam envolvidos na etiologia da depressão.

1.2. Envolvimento dos receptores NMDA e da via L-arginina-Óxido Nítrico na fisiopatologia da Depressão

Evidências têm mostrado que, além do sistema monoaminérgico, vários outros alvos têm sido implicados na patogênese de transtornos depressivos como os receptores glutamatérgicos do tipo N-metil-D-aspartato (NMDA) (Skolnick, 1999) e a via da L-arginina-Óxido Nítrico (NO)-guanosina monofosfato cíclico (GMPc) (Harkin et al., 1999). Além disso, estudos bioquímicos (Nowak et al., 1993), eletrofisiológicos (Bobula et al., 2003) e comportamentais (Popik et al., 2000) têm demostrado que o aumento da neurotransmissão monoaminérgica mediado pelos antidepressivos está associado com uma hipofunção dos receptores NMDA (Zomkowski et al., 2010) e com uma redução da síntese de NO (Krass et al., 2011).

O glutamato é o principal neurotransmissor excitatório do Sistema Nervoso Central – SNC, fundamental na regulação da plasticidade sináptica, crescimento e diferenciação celular. Esse neurotransmissor exerce suas ações através da estimulação de receptores específicos, classificados com base em características estruturais e farmacológicas em metabotrópicos ou ionotrópicos, estes últimos incluem o NMDA, AMPA e cainato. Os receptores NMDA são canais iônicos com uma alta permeabilidade aos íons cálcio que podem ser modulados por ligantes e por voltagem. No estado de repouso, apresentam a ligação de um íon magnésio no interior do canal,

bloqueando a passagem da corrente iônica. Mediante despolarização, geralmente obtida pela ativação prévia de receptores AMPA e influxo de sódio, o magnésio é deslocado e o canal desbloqueado, permitindo o influxo de cálcio (Lau e Tymianski, 2010; Molinoff et al., 1994). No entanto, um aumento da atividade glutamatérgica leva à excitotoxicidade (**Figura 2**) sendo freqüentemente associado a condições patológicas, como epilepsia, doença de Alzheimer, esquizofrenia e depressão (Kornhuber e Weller, 1997; Lau e Tymianski, 2010; Skolnick, 1999).

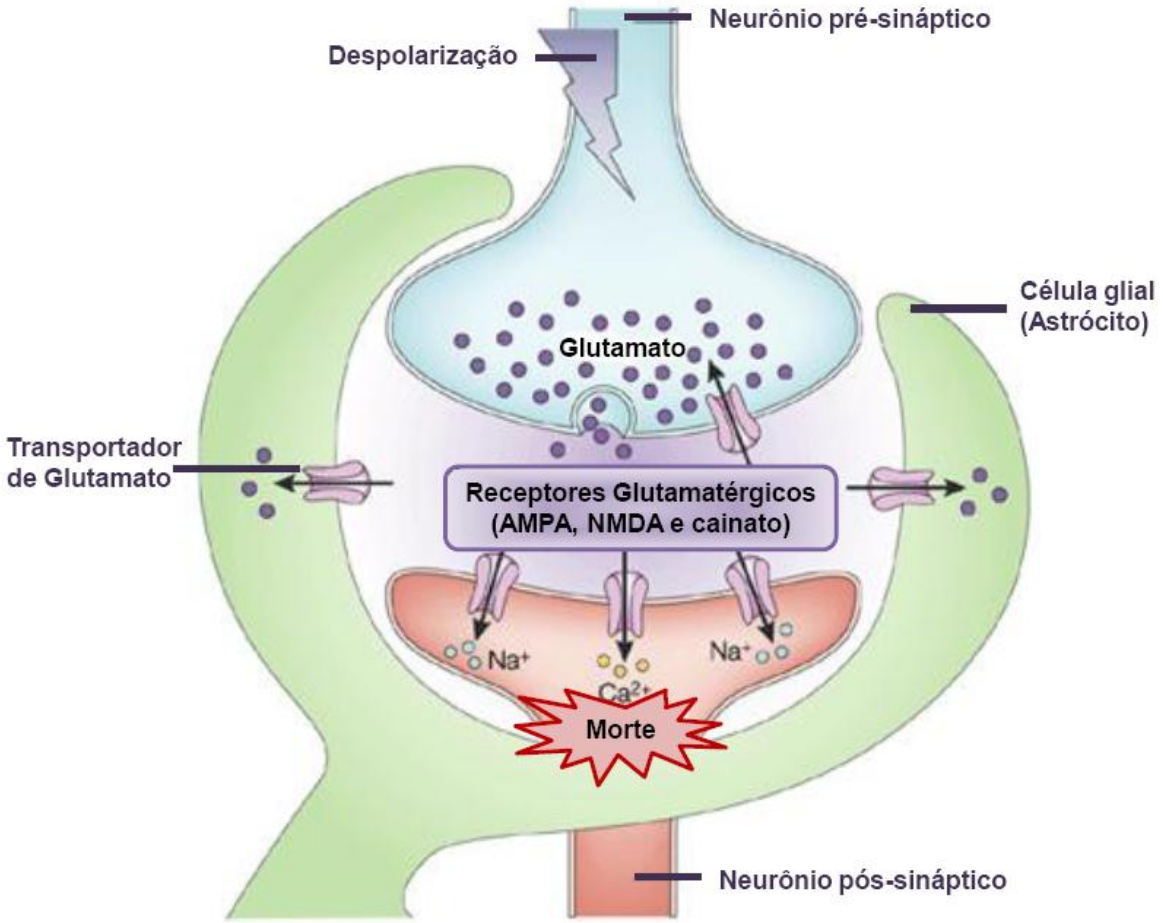

Figura 2. Morte neuronal por excitotoxicidade glutamatérgica. O glutamato é liberado na fenda sináptica por despolarização após um potencial de ação. Os níveis deste neurotransmissor são cuidadosamente controlados por transportadores astrocitários que o captam, reduzindo seus efeitos pós-sinápticos. No entanto, muitas condições deletérias podem convergir para a exocitose desenfreada de glutamato iniciando uma cascata de eventos que leva à excitotoxicidade. O influxo de íons Ca^{+2} intracelular através da ativação exacerbada de receptores glutamatérgicos ionotrópicos AMPA, NMDA e cainato ativa vias de sinalização que culminam na morte celular. (Adaptado de Syntichaki e Tavernarakis, 2003).

A neurotransmissão glutamatérgica pode estar alterada nos transtornos de humor e evidências da literatura têm demonstrado que os antidepressivos também exercem suas ações através da inibição de receptores NMDA (Skolnick, 1999; Szasz et al., 2007). Além disso, moduladores glutamatérgicos, incluindo antagonistas dos receptores NMDA possuem propriedades antidepressivas em modelos animais, como o estresse inescapável **(**Trullas e Skolnick, 1990), o estresse crônico moderado (Papp e Moryl,

1994), o teste do nado forçado (TNF) (Rosa et al., 2003) e o teste da suspensão pela cauda (TSC) (Moretti et al., 2011). Adicionalmente, a administração de antagonistas de receptores NMDA mimetiza o efeito de antidepressivos, revertendo a anedonia induzida pelo estresse crônico moderado (Papp e Moryl, 1994; Skolnick et al., 2009) e potencializa a atividade da fluoxetina, venlafaxina e imipramina no TNF (Pruus et al., 2010; Rogoz et al., 2002). Além disso, Riluzol, um fármaco que aumenta a captação de glutamato pelos astrócitos mostrou-se eficaz como monoterapia e como coadjuvante no tratamento de depressão refratária (Zarate et al., 2004). Cetamina, um antagonista não competitivo do receptor NMDA, produz um efeito antidepressivo rápido e persistente em estudos clínicos (Berman et al., 2000; Kudoh et al., 2002).

O influxo de cálcio, através da ativação de receptores NMDA, induz a ativação da enzima óxido nítrico sintase (NOS). A NOS ativada então converte L-arginina em NO e L-citrulina (Moncada et al., 1989). O NO, uma molécula mensageira no cérebro tem sido implicado na fisiopatologia da depressão (Harkin et al., 1999; Da Silva et al., 2000). Nos neurônios, o melhor alvo caracterizado para o NO é a enzima guanilato ciclase solúvel (GCs), que quando ativada produz o segundo mensageiro intracelular guanosina 3'5'-monofosfato cíclico (GMPc) através de guanosina 5'-trifosfato (GTP). O GMPc é substrato para a enzima fosfodiesterase 5 (PDE_5) que o degrada a guanosina 5'-monofosfato (GMP) (Moncada et al., 1989) (**Figura 3**).

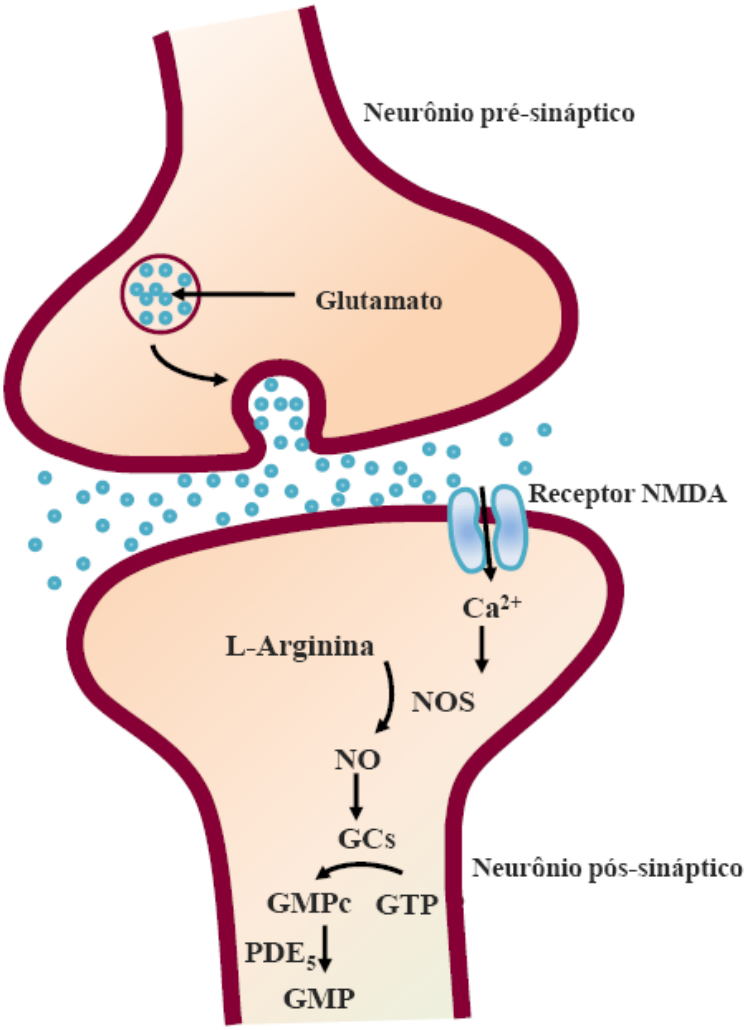

Figura 3. Síntese e metabolismo do NO. A ativação de receptores NMDA promove influxo de cálcio intracelular e ativação da enzima NOS neuronal. A NOS ativada converte L-arginina em NO e L-citrulina**.** O NO, por sua vez, ativa a enzima GCs, que converte GTP a GMPc. O GMPc é substrato para a enzima PDE_5 que o degrada a GMP.

A literatura aponta que os inibidores da NOS exercem efeitos antidepressivos (Da Silva et al., 2000; Harvey et al., 2010). Além disso, o tratamento com inibidores da NOS aumenta a liberação de serotonina no córtex frontal de ratos (Smith e Whitton, 2000) e a serotonina endógena parece ser importante para o efeito antidepressivo de inibidores da NOS, como o 7-nitroindazol, por exemplo (Gigliucci et al., 2010; Harkin et al., 2003). Adicionalmente, estudos in vitro demonstram que o tratamento com antidepressivos clássicos como citalopram, paroxetina e imipramina inibe a atividade da NOS na região hipocampal (Harvey et al., 2010; Wegener et al., 2003).

1.3. Tratamento farmacológico para depressão e sinalização celular

Há um número substancial de farmacoterapias empregadas na clínica para o tratamento da depressão, como os antidepressivos tricíclicos, os inibidores da monoamina oxidase (iMAO), os inibidores seletivos da recaptação de serotonina (ISRS) e os inibidores mistos da recaptação de serotonina e noradrenalina (ISRSN). O tratamento com antidepressivos restabelece a falha no processamento de informações em algumas redes neuronais no cérebro dos pacientes deprimidos (**Figura 4**).

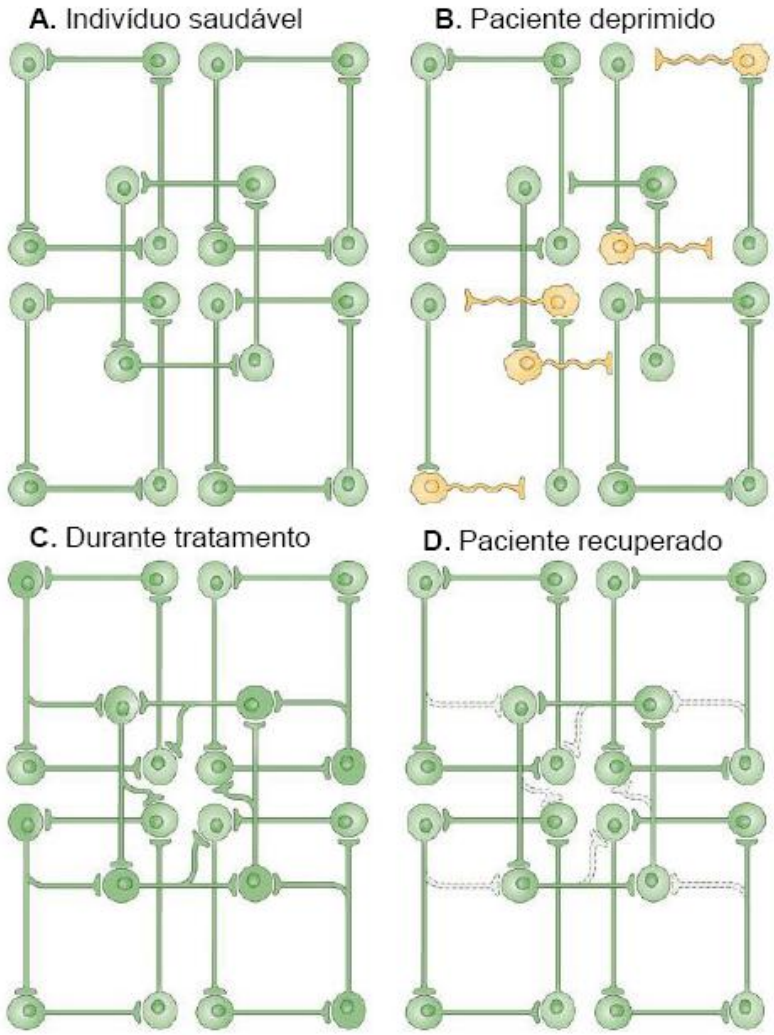

Figura 4. Os antidepressivos restabelecem as sinapses e redes neuronais deficientes. A. No cérebro saudável, as informações são processadas em redes neurais que se sobrepõem parcialmente. **B.** Nos estados depressivos observa-se que o processamento de informações em algumas redes neuronais não funciona corretamente. **C.** O tratamento com antidepressivo melhora a conectividade das redes neurais. **D.** Após o tratamento e recuperação do paciente, as sinapses e redes neuronais ativas são selecionadas e estabilizadas (Adaptado de Castrén, 2005).

O tratamento crônico, e não agudo, com antidepressivos aumenta o nível de proliferação neuronal hipocampal em adultos (Sairanen et al., 2005). Estes achados corroboram com a hipótese neurotrófica da depressão que postula que o tratamento com antidepressivos estimula a atividade neuronal e promove aumento da expressão do fator neurotrófico derivado do cérebro (BDNF) (Russo-Neustadt and Chen, 2005). BDNF ativa uma variedade de cascatas de sinalização, incluindo a fosfatidilinositol 3'-cinase

(PI-3K)-Akt (Numakawa et al., 2010), a via da proteína cinase ativada por mitógeno (MAPK) (Chen et al., 2007). Essas vias de sinalização, assim como outras que promovem sobrevivência neuronal, convergem em um regulador trascripcional, a proteína de ligação responsiva ao AMP cíclico (AMPc) (CREB). Uma variedade de fatores de crescimento e hormônios estimula a expressão de genes celulares quando CREB é ativado por fosforilação em Ser^{133} (Tardito et al., 2006). Originalmente caracterizado como um alvo de fosforilação da proteína cinase A (PKA), atualmente sabe-se que outras cinases também são responsáveis pela ativação mediada por fosforilação de CREB como a proteína cinase dependente de Ca^{+2}/calmodulina (CaM) e a via Ras-Raf-MAP cinase (MEK)-ERK que catalisa a transferência de um grupo fosfato do ATP para resíduos Ser^{133} através de RSK2, um membro da família cinase ribossomal S6 (Nair e Vaidya, 2006). CREB quando ativado liga-se ao elemento de resposta ao AMPc (CRE) no DNA para regular a expressão gênica de alvos como neurotrofinas e fatores tróficos (BDNF) que contribuem para a neuroplasticidade e modulação do humor mediadas pelo tratamento crônico com antidepressivos (**Figura 5**).

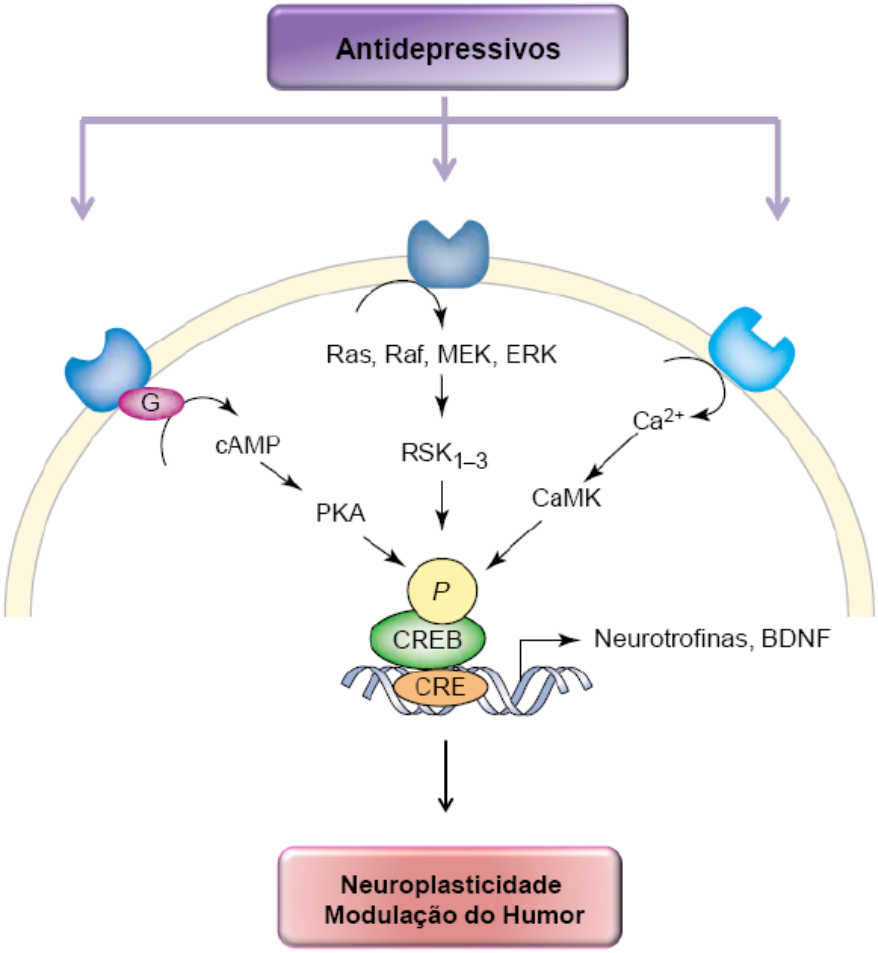

Figura 5. Regulação da proteína de ligação responsiva ao AMPc (CREB) pelo tratamento crônico com antidepressivos. Grande parte dos antidepressivos modula a neurotransmissão monoaminérgica. A ligação destes neurotrasmissores a receptores neuronais ativa segundos mensageiros intracelulares como AMPc, Ca^{2+} e cinases como PKA, CaMK, MEK, ERK e proteínas cinase ribossomais S6. Estas cinases fosforilam proteínas substratos como o fator de transcripção CREB. CREB ativado se liga ao elemento de resposta ao AMPc (CRE) no DNA para regular a expressão gênica de neurotrofinas e fatores tórficos (BDNF) resultando neuroplasticidade e modulação do humor (Adaptado de Malberg e Blendy, 2005).

A habilidade dos antidepressivos em ativar proteínas cinases celulares que fosforilam CREB mostra a ativação deste fator de transcrição como parte do mecanismo de ação destes medicamentos. De fato, a administração crônica de fluoxetina em ratos aumenta a fosforilação de CREB (pCREB) em várias regiões do cérebro, incluindo a amígdala, córtex, giro denteado e hipotálamo (Nair e Vaidya, 2006).

Recentemente, o envolvimento da família das MAPKs nos mecanismos moleculares da depressão está crescendo, e evidências têm mostrado que a ERK pode participar da modulação neuronal da depressão (Fumagalli et al., 2005; Todorovic et al. 2009). Entre as MAPKs, as cinases 1 e 2 reguladas por sinal extracelular (ERK1 e ERK2) são as mais bem caracterizadas. A via da ERK é o principal ponto de convergência em todas as vias de sinalização, regulando apoptose, crescimento celular, diferenciação e neuroplasticidade (Johnson and Lapadat, 2002; Strnisková et al., 2002). ERK1/2 é ativada através da ligação do BDNF a seu receptor tropomiosina cinase B (TrkB) pela cascata MEK-ERK induzindo translocação nuclear e fosforilação de fatores de trasncrição.

Adicionalmente, várias linhas de evidências têm mostrado que transtornos depressivos estão relacionados com ativação da enzima glicogênio sintase cinase-3β (GSK-3β), através da falha em seu mecanismo de inibição mediado por fosforilação (Beaulieu et al., 2009). Várias cinases como a Akt/proteína cinase B (PKB), a proteína cinase A (PKA) e a proteína cinase C (PKC) regulam negativamente a atividade da enzima GSK-3β por fosforilação na região N-terminal de Ser^{9} (Beaulieu et al., 2009). Akt/PKB é uma serina/treonina cinase que fosforila e regula a função de muitas proteínas celulares envolvidas em processos relacionados ao metabolismo, apoptose, proliferação e neuroplasticidade (Song et al., 2005), incluindo GSK-3β. Akt/PKB contém dois sítios regulatórios de fosforilação, o Thr^{308} no domínio catalítico e Ser^{473} no domínio regulatório C-terminal (Nicholson e Anderson, 2002). GSK-3β é uma serina/treonina cinase multifuncional e muito ativa que recebeu este nome após a descoberta de seu envolvimento no metabolismo do glicogênio (Jope and Roh, 2006). GSK-3 (isoformas α e β) é um importante regulador da síntese de glicogênio, transcrição de genes, plasticidade sináptica e apoptose (morte celular) (Jope, 2003). Está

bem estabelecido na literatura que a GSK-3 regula o comportamento, afetando a β-catenina, receptores glutamatérgicos, os ritmos circadianos e a neurotransmissão serotoninérgica (Beaulieu et al., 2008). Todos estes alvos têm sido implicados na fisiopatologia dos transtornos de humor e estudos têm mostrado que inibidores da GSK-3β podem vir a se constituir em uma nova classe de fármacos antidepressivos (Rosa et al., 2008; Du et al., 2010).

1.4. Bulbectomia Olfatória Bilateral

O efeito antidepressivo de diferente classes de compostos em ensaios pré-clínicos é comumente avaliado pelo TNF (Cryan et al., 2005) e TSC (Steru et al., 1985). A principal limitação destes modelos de estresse inescapável é que os animais não estão de fato com sintomas de depressão e que a atividade antidepressiva já é detectada após o tratamento agudo, entretando na prática clínica, cerca de 3-4 semanas são necessárias para que os efeitos terapêuticos sejam observados (Nestler et al., 2002). Uma exceção a esta regra é o modelo de indução de depressão através da Bulbectomia Olfatória (BO) bilateral que é capaz de detectar a atividade antidepressiva de fármacos quase que exclusivamente após tratamento crônico dos mesmos (Leonard, 1984). A BO induz alterações comportamentais que mimetizam vários sintomas de depressão como disfunções de aprendizado e memória, agitação psicomotora e anedonia (Kelly et al., 1997).

Estudos clínicos reportam que ativações anormais em diversas regiões cerebrais na depressão, incluindo a amígdala, cíngulo anterior e córtex pré-frontal, podem estar associadas a alterações na percepção olfatória destes pacientes (Song e Leonard, 2005; Lombion e Pouthier, 2006; Pollatos et al., 2007). O sistema olfatório desempenha funções importantes na fisiologia e comportamento dos mamíferos. Além da importância da sensação de odor, a olfação também exerce influência na regulação do

consumo de alimentos e interfere no comportamento social, sexual e materno (Leonard, 1984; Meguid et al., 1997; González-Mariscal et al., 2003). A ablação bilateral dos bulbos olfatórios resulta em alterações comportamentais, neuroquímicas, neuroendócrinas, neuroimunológicas e morfológicas, características compatíveis e comparáveis ao que é observado em pacientes com depressão (Jesberger e Richardson, 1988; Cryan e Mombereau, 2004; Song e Leonard, 2005).

Os bulbos olfatórios são extensões bilaterais do telencéfalo rostral e constituem cerca de 4% de toda massa cérebral de um rato adulto. Devido às extensas conexões eferentes com o cérebro mesocortical e regiões subcorticais límbicas, a remoção dos bulbos olfatórios tem grande impacto sobre as funções cerebrais e modulação do humor (Song e Leonard, 2005). Os efeitos comportamentais induzidos pela BO não são conseqüências da anosmia em si, uma vez que a destruição de receptores sensoriais olfatórios não induz sintomas depressivos (Alberts and Friedman, 1972; Saitoh et al., 2006). Desta forma, a bulbectomia olfatória (BO) em roedores tem sido proposta como um modelo animal de depressão (Leonard, 1984; Song e Leonard, 2005).

1.5. *Tabebuia avellanedae*

A farmacoterapia atua disponível para o manejo da depressão, apesar de amplamente utilizada, mostra-se pouco eficaz, não ultrapassando 60% de eficácia (Kiss, 2008; Nestler et al., 2002), seus efeitos clínicos são observados somente após cerca de 3-4 semanas do início do tratamento, e possui baixa tolerabilidade em virtude de seus efeitos adversos, como: hipotensão, arritmia, insônia e ganho de peso (Morilak e Frazer, 2007). Desta maneira, esforços consideráveis são investidos na busca de melhores fármacos e/ou tratamentos combinados para a depressão.

Tabebuia avellanedae Lorentz ex Griseb (sinonímia científica *Tabebuia*

impetiginosa Martius ex DC) (Bignoniaceae) é uma árvore nativa das florestas tropicais brasileira conhecida por sua copa sempre verde e pela beleza de suas flores roxas. A sinonímia *T. impetiginosa* é reminiscente do uso popular desta planta no tratamento de impetigo (Lüebeck, 1999). *T. avellanedae* já foi catalogada em outros países da América do Sul como Argentina, Bolívia, Colômbia, Equador, Paraguai, Peru e Venezuela (Lüebeck, 1999; Taylor, 2005). Popularmente chamada de "ipê-roxo", por suas folhas roxas, e "pau d'arco", pelo costume indígena de confeccionar arcos com sua madeira, o chá das cascas do tronco de *T. avellanedae* possui vários usos etnofarmacológicos pelo povo indígena que a denominada de árvore divina (Lüebeck, 1999). Os usos etnofarmacológicos de *T. avellanedae* são anteriores à descoberta do Brasil – segundo relatos dos primeiros portugueses que chegaram a nosso país, a tribo indígena Guarani já utilizava chá das cascas desta planta de maneira abundante para malária, câncer, febre, tripanossomíase, desordens estomacais, infecções fúngicas e bacterianas, como tônico energizante e para o alívio de uma variedade de estados mentais e emocionais, como ansiedade, falta de memória, irritabilidade e depressão (Lüebeck, 1999; Gómez Castellanos et al., 2009).

Os principais compostos fitoquímicos isolados da casca de *T. avellanedae* são flavonóides, dialdeídos ciclopentênicos, ácidos benzóicos e derivados benzoaldeídos, quinonas, furanonaftoquinonas e os mais importantes – naftoquinonas e antraquinonas. Entre as 18 quinonas mais relevantes registradas até agora, Lapachol e β-Lapachona parecem ser os de maior importância clínica uma vez que têm sido relacionadas com as atividades farmacológicas do chá de *T. avellanedae* (Gómez Castellanos et al., 2009).

Após uma série de relatos de efeitos positivos e "milagrosos" após o uso popular do chá de *T. avellanedae* no tratamento de câncer, entre os anos 1960 e 1990, o Instituto Nacional de Câncer dos Estados Unidos se interessou em pesquisar os efeitos

antitumorais desta planta (Gómez Castellanos et al., 2009) e em 1999 o FDA (Administração de Drogas e Alimentos dos Estados Unidos) registrou o uso do chá de *T. avellanedae* como suplemento dietético para alivio dos estados e sintomas de câncer (FDA, 1999).

De fato, estudos mostraram que quinonas (antraquinonas e naftoquinonas) possuem propriedades antitumorais e antimicrobianas (O'Brien, 1991; Boik, 2001). Adicionalmente, outras propriedades farmacológicas da *T.avellanedae* foram descritas como: antibacteriana (Park et al., 2005), antiagregante plaquetária (Son et al., 2006), antinociceptiva, antiedematogênica (De Miranda et al., 2001), antiulcerogênica (Twardowschy et al., 2008), antiangiogênica (Kung et al., 2007), antinflammatória (Byeon et al., 2008), antifúngica (Portillo, et al., 2001), diurética (Byeon et al., 2008) e antipsórica (Müller et al., 1999). Recentemente, nosso grupo demonstrou que o extrato etanólico obtido das cascas dessa planta, quando administrado sistemicamente a camundongos produz um efeito do tipo antidepressivo específico em modelos preditivos de atividade antidepressiva – TNF e TSC (Freitas et al., 2010). Além disso, verificou-se que este efeito é mediado, pelo menos em parte, pela interação com os sistemas serotoninérgico, noradrenérgico e dopaminérgico. Adicionalmente, observou-se que a coadministração de doses sub-efetivas de extrato e de antidepressivos clássicos (fluoxetina, desipramina e bupropiona) foi efetiva na produção de um efeito do tipo-antidepressivo em camundongos (Freitas et al., 2010)

1.6. Fluoxetina

A fluoxetina é um antidepressivo da classe dos ISRS largamente empregado no tratamento de depressão, transtorno obsessivo compulsivo, bulimia e transtorno de pânico (Mostert et al., 2008). Este medicamento foi aprovado pelo FDA para o

tratamento da depressão em 1987 (Wong et al., 2005). O alvo dos ISRS é o transportador de serotonina (5-HTT), que está localizado na membrana plasmática de neurônios serotoninérgicos, e é responsável pela recaptação de 5-HT com alta afinidade. A inibição do transportador 5-HTT resulta aumento significativo dos níveis de 5-HT extracelulares, e assim, ativa de maneira sustentada receptores 5-HT pré e pós-sinápticos. No cérebro, a 5-HT é sintetizada a partir do aminoácido triptofano obtido através da dieta, exclusivamente em neurônios localizados nos núcleos da rafe, de onde partem projeções para todas as partes do SNC (Wong et al., 2005). Os receptores 5-HT são classificados em diferentes famílias (1, 2, 3, 4, 5, 6 e 7) e são capazes de ativar diferentes mecanismos de transdução de sinais (Barnes e Sharp, 1999, Nichols e Nichols, 2008). Os neurônios serotoninérgicos enviam axônios para diversas regiões cerebrais responsáveis pelo controle da emoção, aprendizado, memória, comportamento alimentar, sono e vigília e comportamento sexual (Mostert et al., 2008).

Importantes funções celulares envolvidas em sobrevida neuronal e neuroplasticidade são moduladas pelo tratamento com fluoxetina, destacando-se: regulação do fator de transcrição CREB, produção de fatores neurotróficos (BDNF) e da proteína astrocitária S100β, regulação do suprimento de energia, abertura e fechamento de canais iônicos e diminuição da condutância de canais mitocondriais voltagem dependente (Mostert et al., 2008).

Estudos têm demonstrado que o tratamento com fluoxetina produz efeito do tipo antidepressivo em modelos animais de depressão como o estresse crônico moderado (First et al., 2011), o estresse crônico de contenção (Christiansen et al., 2011), a bulbectomia olfatória bilateral (Mar et al., 2002), o comportamento depressivo induzido pela administração de corticosterona (Rainer et al., 2011) e pelo fator de necrose tumoral (TNF-α) (Kaster et al., 2012). A avaliação do efeito causado por

antidepressivos clássicos e por possíveis compostos antidepressivos nestes modelos é relevante para a elucidaçao dos mecanismos envolvidos na ação antidepressiva destas substâncias.

2. Justificativa

Segundo a Associação Americana de Psiquiatria (2000), a agitação ou retardo psicomotor e a anedonia são elementos utilizados como critério para o diagnóstico de episódios de depressão. Em ambos manuais diagnósticos, a agitação psicomotora pode estar presente tanto na depressão unipolar quanto bipolar, porém a distinção no diagnóstico clínico entre agitação e retardo ainda não é bem estabelecido. Historicamente, a "depressão agitada" foi primeiramente considerada como um subtipo de melancolia (agitação melancólica) e depois passou a ser associada com estados de ansiedade (Angst et al., 2009; Mitchell e Malhi, 2004).

Esta característica marcante de hiperatividade é acessível através do modelo da bulbectomia olfatória e não em outros modelos que induzem comportamento tipo-depressivo ou em testes preditivos. Outra característica marcante deste modelo corresponde ao fato que as alterações comportamentais e/ou bioquímicas desencadeadas pela ablação dos bulbos olfatórios somente serem revertidas com o tratamento crônico e não agudo com antidepressivos. Adicionalmente, a BO em roedores reproduz sintomas de anedonia (perda de interesse ou satisfação em quase todas as atividades) e apatia apresentada pelos pacientes deprimidos (Willner, 2005). Por estes motivos, selecionou-se este modelo, pois além de outras alterações desencadeadas as mais relevantes são a hiperatividade, a anedonia e o fator temporal da atuação dos antidepressivos que corresponde ao que é observado na clínica (Lumia et al., 1992).

Adicionalmente, Gary e seu grupo (2002) mostraram que ratos submetidos à BO apresentam uma diminuição da vulnerabilidade dos neurônios piramidais do hipocampo à lesão causada por excitotoxicidade, sugerindo que a ablação dos bulbos afeta fatores neurotróficos nesta região cerebral. Considerando o fato de que os mecanismos celulares e moleculares subjacentes a esta neuroplasticidade em resposta à BO são pouco estudados, justifica-se a elucidação dos efeitos da BO na modulação de vias de sinalização associadas à plasticidade sináptica bem como a habilidade do extrato de *Tabebuia avellanedae* e/ou da fluoxetina de reverterem tais alterações na região hipocampal.

Em resumo, o modelo é proposto como adequado para representar um episódio de depressão caracterizada pela agitação psicomotora e anedonia crônica. Sendo assim, a maior característica comportamental deste modelo é a resposta de hiperatividade locomotora e que esta é exclusivamente revertida pelo tratamento crônico e não agudo com antidepressivos (Hellweg et al., 2007). Desta maneira, acredita-se que se o extrato de *Tabebuia avellanedae* quando administrado sistemicamente já exibiu um efeito antidepressivo nos testes preditivos (teste do nado forçado e teste da suspensão da cauda), esse composto também possa a vir a ter efeitos positivos em modelos que induzam comportamento tipo-depressivo (no presente estudo a BO), apresentando desta maneira, maiores perspectivas para ser utilizado futuramente em modelos clínicos e podendo vir a se tornar uma nova alternativa terapêutica para a depressão.

Em paralelo a este contexto, está a caracterização do mecanismo de ação antidepressiva do extrato de *Tabebuia avellanedae* através da investigação da participação do sistema glutamatérgico (via receptores NMDA) e da via da L-arginina-NO/GMP.

Apesar de o glutamato ser o principal neurotransmissor excitatório do SNC, fundamental na regulação da plasticidade sináptica, crescimento e diferenciação celular, sabe-se que um aumento da atividade glutamatérgica leva à excitotoxicidade sendo freqüentemente associado a condições patológicas, incluindo a depressão (Kornhuber e Weller, 1997; Lau e Tymianski, 2010; Sanacora et al., 2008). Além disso, estudos pré-clínicos demonstram que antagonistas de receptores glutamatérgicos do tipo NMDA possuem propriedades antidepressivas em modelos animais, como o estresse crônico, desamparo aprendido, e o próprio modelo de bulbectomia olfatória e de desespero comportamental, como o teste do nado forçado (TNF) e o teste da suspensão pela cauda (TSC) (Skolnick, 1999; Kugaya e Sanacora, 2005).

Adicionalmente, a literatura afirma que apesar de desempenhar um papel relevante na sinalização neuronal e plasticidade sináptica (Esplugues, 2002; Silverman, 2009), o NO está implicado na morte neuronal induzida por glutamato (Strijbos et al., 1996) e que os inibidores da NOS e da GCs produzem efeito antidepressivo em modelos animais (Harkin et al., 2003; Kaster et al., 2005), assim como antidepressivos clássicos diminuem a atividade da NOS no hipocampo (Wegener et al., 2003). Sendo assim, o presente projeto se propôs a avaliar a participação do sistema glutamatérgico (via receptores NMDA) e da via da L-arginina-NO/GMPc na acão antidepressiva do extrato de *Tabebuia avellanedae* no TSC.

3. Objetivos

3.1. Objetivo geral

Investigar possíveis mecanismos de ação envolvidos no efeito antidepressivo do extrato de *Tabebuia avellanedae*

3.2. Objetivos específicos

- Verificar o efeito do tratamento repetido (14 dias) com extrato de *T. avellanedae* administrado por via oral (p.o.) no modelo preditivo de atividade antidepressiva: teste da suspensão pela cauda (TSC) e sobre a atividade locomotora dos animais no teste do campo aberto (TCA).
- Caracterizar o modelo da BO do ponto de vista das alterações comportamentais e neuroquímicas que acarreta aos camundongos, validando-o assim para as condições do laboratório como um modelo de indução de comportamento tipo-depressivo.
- Investigar o efeito do extrato de *T. avellanedae* e da fluoxetina sobre o aumento da atividade locomotora no TCA de camundongos submetidos à BO.
- Monitorar o comportamento dos animais bulbectomizados no splash teste, com o intuito de observar se a BO induz um comportamento anedônico e se este é revertido pelo tratamento com o extrato de *T. avellanedae* ou fluoxetina.
- Analisar amostras do hipocampo de animais operados (BO) e não operados (Sham), e que foram tratados de forma repetida com extrato de *T. avellanedae* ou fluoxetina, a fim de se verificar e comparar a expressão de proteínas envolvidas na sobrevivência celular (pAkt, pGSK-3β, pERK1/2, pCREB e fator neurotrófico BDNF).
- Investigar a participação do sistema glutamatérgico (via receptores NMDA) e da via da L-arginina-NO/GMPc na ação antidepressiva do extrato de *T. avellanedae* no TSC.

4. Materiais e Métodos

4.1. Animais

Foram utilizados camundongos *Swiss* adultos fêmeas, pesando entre 35 e 45g e mantidos a 20 ± 2°C com livre acesso a água e comida, em ciclo claro/escuro 12:12 horas (7:00-19:00 h). Os animais foram fornecidos pelo Biotério Central da Universidade Federal de Santa Catarina e mantidos no biotério setorial nas mesmas condições. Todos os procedimentos realizados foram aprovados pela Comissão de Ética no Uso de Animais da UFSC (CEUA) e todos os esforços foram feitos para minimizar o sofrimento dos animais.

4.2. Bulbectomia olfatória

Depois de um período de ambientação de 2 semanas, a BO bilateral foi realizada pelo método da sucção, descrito previamente por Leonard e Tuite (1981). Os animais foram divididos aleatoriamente em dois grupos experimentais: BO e Sham. Os camundongos foram anestesiados com xilazina (6 mg/kg, i.p.) em combinação com quetamina (100 mg / kg, ip) diluídos em solução salina. Uma incisão na pele que cobre o crânio foi realizada, e após a exposição do crânio, os orifícios foram perfurados em ambos os lados da linha média. Em seguida, os bulbos olfatórios foram bilateralmente aspirados com o auxílio de uma agulha hipodérmica (1,0-1,2 cm de comprimento com uma ponta arredondada de 0,80 a 1,2 mm de diâmetro) acoplada a uma seringa (10 mL), tomando cautela para não causar nenhuma lesão ao córtex frontal. Sangramentos foram cessados, os orifícios limpos com algodão e cobertos com resina dental. Todo o procedimento cirúrgico foi realizado utilizando álcool 70% para eliminarem quaisquer possíveis contaminações. Os animais Sham foram submetidos ao mesmo protocolo, porém sem ablação dos bulbos olfatórios. A temperatura corpórea dos animais foi

controlada com auxílio de aquecimento artificial. Após a cirurgia, os animais passaram por um período de 14 dias de recuperação antes do início dos tratamentos. A porcentagem de erro do procedimento cirúrgico foi cerca de 17%.

4.3. Drogas e Tratamentos

4.3.1. Material vegetal e preparação do extrato etanólico de T. avellanedae

Cascas de *T. avellanedae* foram fornecidas pela Chamel Indústria e Comércio de Produtos Naturais Ltda (Campo Largo, Brasil), lote 4753. A identificação foi realizada pelo botânico Elide Pereira dos Santos e uma amostra foi depositada no Herbárium do Departamento de Botânica da Universidade Federal do Paraná (UFPR), Brasil. Cascas secas e em pó (5 kg) foram extraídos por maceração com etanol a 95% por 7 dias à temperatura ambiente. O extrato etanólico foi filtrado, o solvente evaporado sob pressão reduzida (40-50°C), liofilizado e como produto final obteve-se um composto sólido vermelho-marrom (919,2 g).

4.3.2. Caracterização fitoquímica do extrato etanólico de T. avellanedae por Eletroferose capilar

A análise do extrato etanólico de *T. avellanedae* foi realizada em um sistema de eletroforese capilar (CE) (HP3DCE, Agilent Technologies, Palo Alto, CA, USA) equipado com um detector de diodo (200 nm). A análise foi conduzida a 25°C em um capilar de sílica (48.5 cm×50 μm I.D.×375 μm O.D.) obtido de Polymicro (Phoenix, AZ, USA). No primeiro condicionamento, o capilar foi lavado durante 30 min. com hidróxido de sódio 1,0 M seguido de água deionizada (30 min.). Entre as corridas, os capilares foram secados por 5 min. com eletrólito de corrida (tetraborato de sódio 20 mmol L-1 e metanol 10%, pH 9,0). As soluções padrão e amostra foram introduzidas a partir da extremidade de entrada do capilar e injetadas de maneira hidrodinâmica a 50

mbar (50 mbar=4996.2 Pa) durate 6 s. Foi aplicada uma voltagem de separação de 30 kV, com a polaridade positiva voltada para o lado da injeção. Ácido caféico (100 mg L-1) foi utilizado como padrão-interno e detectado a 300 nm.

Preparação da amostra: 0,5299 g de extrato etanólico foi solubilizado em 10 mL de solução metanol:água 50% (v/v).

4.3.3. Investigação do efeito do tratamento repetido (14 dias) com extrato de T. avellanedae e com fluoxetina nos animais submetidos ao modelo da Bulbectomia Olfatória

Para se verificar a dose ideal de extrato de *T. avellanedae* para o tratamento dos animais bulbectomizados, primeiramente, uma curva dose-resposta com o extrato foi realizada. Para este fim, os animais foram tratados com doses crescentes de extrato de *T. avellanedae* (10, 30 ou 100 mg/kg) por via oral (gavagem) durante 14 dias, e após 24 h do último tratamento os mesmos foram submetidos ao TSC. A fim de se analisar o efeito deste tratamento sobre a atividade locomotora dos animais, o mesmo procedimento foi realizado, e os animais testados no TCA.

Posteriormente, em uma nova seqüência de experimentos, os animais foram tratados com extrato (10-30 mg/kg, p.o.) ou fluoxetina (Sigma Chemical Co., 10 mg/kg, p.o.) após 14 dias de recuperação (pós-operatório) da BO. Após o tratamento repetido (14 dias), os camundongos foram testados após 24 horas do último tratamento, no TCA e demais testes comportamentais. O grupo controle (Sham) recebeu veículo (água ou água contendo no máximo 5 % de Tween 80). Os animais foram divididos nos seguintes grupos experimentais: (a) sham/veículo, (b) sham/extrato 10 mg/kg, (c) sham/extrato 30 mg/kg e (d) sham/fluoxetina 10 mg/kg, grupos controle; (e) BO/veículo, (f) BO/extrato 10 mg/kg, (g) BO/extrato 30 mg/kg, (h) BO/fluoxetina 10 mg/kg. Foram utilizados 9-12 animais/grupo.

4.3.4. Investigação do envolvimento da via L-arginina-Óxido Nítrico e dos receptores NMDA na ação antidepressiva do extrato de T. avellanedae

Os seguintes compostos foram utilizados: L-arginina, NMDA (N-methyl-D-aspartato), (1H-[1,2,4]oxadiazol[4,3-a]quinoxalin-1-one) (ODQ), 7-nitroindazol (Sigma Chemical Co, USA), MK-801 (RBI, Boston, MA, USA) e sildenafil (Pfizer). Os fármacos foram diluídos em salina (NaCl 0,9%) exceto ODQ, que foi dissolvido em salina com 1% de DMSO e 7-nitroindazol que foi dissolvido em salina com algumas gotas de Tween 80. Os grupos controle receberam o veículo apropriado. A via de administração utilizada foi a i.p., exceto o NMDA e ODQ, que foram administrados por via i.c.v.

Para administração i.c.v. foi utilizada uma agulha de 0,4 mm de diâmetro conectada por uma cânula de propileno a uma seringa Hamilton de 25 µl. A agulha foi inserida perpendicularmente no crânio, diretamente no ventrículo lateral, utilizando-se o bregma como referência (1 mm lateral e 1 mm posterior ao bregma, com uma perfuração de 2,4 mm de profundidade). A fim de se verificar o local exato da injeção, os animais foram dissecados e analisados macroscopicamente após os testes (Brocardo et al., 2008).

O extrato de *T. avellanedae* (1-30 mg/kg) foi dissolvido em água destilada contendo no máximo 5 % de Tween 80 e foi administrado por via oral (p.o.) 60 min antes do TSC ou TCA. O grupo controle recebeu água destilada contendo no máximo 5 % de Tween 80.

Com a finalidade de se investigar a hipótese de que o efeito antidepressivo do extrato de *T. avellanedae* é mediado através da inibição de receptores NMDA, os animais foram pré-tratados com extrato (30 mg/kg, p.o) ou controle e 30 minutos após

receberam NMDA (0,1 pmol/sitio, i.c.v.) ou salina. Decorridos 30 minutos, os animais foram testados no TSC e TCA.

Em outro experimento, a fim de se analisar um efeito sinérgico do extrato de *T. avellanedae* com um antagonista de NMDA, os animais foram pré-tratados com uma dose sub-ativa de MK-801 (0,01 mg/kg, p.o., antagonista não competitivo de receptores NMDA) ou salina, e imediatamente após, receberam uma dose sub-ativa de extrato (1 mg/kg, p.o.) ou controle. Decorridos 60 minutos, os animais foram testados no TSC e TCA.

Posteriormente, para se verificar a participação da via L-arginina-Óxido Nítrico no efeito antidepressivo do extrato de *T. avellanedae* no TSC, os camundongos foram tratados com L-arginina, um precursor do NO (750 mg/kg, i.p., dose que per se não produz efeito do TSC) ou salina, e após 30 minutos os animais receberam extrato (30 mg/kg, p.o.) ou controle. Decorridos 60 minutos, os animais foram submetidos ao TSC e TCA.

No intuito de se investigar um efeito combinado do extrato de *T. avellanedae* com um inibidor da GCs, os animais foram pré-tratados com uma dose sub-efetiva de extrato (1 mg/kg, p.o.) ou controle, e decorridos 30 minutos, os animais receberam uma dose sub-efetiva de ODQ (30 pmol/sítio, i.c.v., um inibidor específico da GCs), ou salina e após 30 minutos foram submetidos ao TSC e TCA.

Para se analisar um efeito sinérgico do extrato de *T. avellanedae* com um inibidor específico da NOS neuronal, os animais foram tratados com uma dose sub-ativa de extrato (1 mg/kg, p.o.) ou controle, e 30 minutos após receberam uma dose sub-ativa

de 7-nitroindazol (25 mg/kg, i.p., um inibidor especifico da NOS neuronal) ou salina, e após 30 minutos os animais foram testados no TSC e TCA.

E por fim, com o objetivo de se verificar a participação do GMPc na ação antidepressiva do extrato de *T. avellanedae*, os camundongos receberam uma injeção de sildenafil (5 mg/kg, i.p., um inibidor da fosfodiesterase 5) ou salina, e após 30 minutos foram tratados com extrato (30 mg/kg, p.o.) ou controle, e decorridos 60 minutos, os animais foram submetidos ao TSC e TCA.

Todos os tratamentos e doses utilizadas foram baseados em estudos prévios do nosso grupo (Da Silva et al., 2000; Mantovani et al., 2003; Kaster et al., 2005; Brocardo et al., 2008; Zomkowski et al., 2010; Moretti et al., 2012).

4.4. Testes comportamentais

4.4.1. Teste do Campo Aberto

A BO é um modelo animal realizado em roedores que reproduz em testes pré-clínicos, a agitação psicomotora evidenciada em alguns pacientes deprimidos (Zueger et al., 2005). A fim de se avaliar a atividade locomotora dos animais submetidos a BO, os camundongos foram submetidos ao teste do campo aberto, como descrito por Rodrigues et al. (1996). Desta forma, o teste foi realizado em três momentos experimentais, primeiro teste antes da BO para verificar a locomoção basal dos camundongos, o segundo teste 2 semanas após a bulbectomia para verificar se o modelo reproduziu a agitação psicomotora nos animais BO, e o terceiro momento após o tratamento repetido dos animais com os compostos abordados neste estudo. Grupos adicionais foram submetidos apenas uma vez ao campo aberto, após o tratamento repetido com extrato. O teste foi realizado em uma caixa de madeira medindo 40 x 60 x 50 cm altura, com o

chão dividido em 12 quadrados iguais. O número de quadrados cruzados com as quatro patas (cruzamentos) foi registrado em uma sessão de 6 minutos.

4.4.2. Teste da Suspensão pela Cauda

O tempo total de duração da imobilidade foi medido de acordo com o método de Steru et al. (1985). Os camundongos acústica e visualmente isolados foram suspensos 50 cm acima do chão por fita adesiva e a imobilidade registrada durante 6 minutos. Os antidepressivos reduzem o tempo de imobilidade neste teste (Steru et al., 1985; Rodrigues et al., 2002; Mantovani et al., 2003; Cryan et al., 2005).

4.4.3. Splash teste

O splash teste foi realizado 24 h após a última administração repetida dos compostos, e adaptado daquele utilizado por Isingrini e colaboradores (2010), com pequenas modificações. O teste consiste na vaporização de uma solução de sacarose a 10% sobre o dorso de um animal colocado individualmente em um aparato de acrílico (9 x 7 x 11cm). O splash teste é um válido marcador comportamental de comportamento anedônico induzido por modelos de depressão, de modo que, após a aplicação de solução de sacarose, o tempo de latência e o tempo total de auto-limpeza são mensurados durante 5 minutos. Este teste avalia o auto-cuidado e o comportamento motivacional dos animais (Willner, 2005; Yalcin et al., 2005).

4.5. Western Blot

Após duas semanas de tratamento, e 24 h após a última administração de extrato de *T. avellanedae* ou de fluoxetina, os animais foram decapitados. Os cérebros foram removidos e a lesão avaliada macroscopicamente; todos os cérebros cuja cirurgia tenha sido incompleta ou com danos ao córtex cerebral foram descartados. Os hipocampos foram rapidamente dissecados e colocados em solução salina, sob refrigeração. O

western blot foi realizado conforme descrito anteriormente (Leal et al, 2002; Cordova et al, 2004). Resumidamente, os tecidos hipocampais foram homogeneizadas mecanicamente em 400 mL de Tris-base 50 mM pH 7,0, EDTA 1 mm, fluoreto de sódio 100 mM, PMSF 0,1 mM, Na_3VO_4 2 mM, Triton X-100 1%, glicerol 10%, e então incubados por 30 min em gelo. Os lisados foram centrifugados (10000 xg por 10 min, a 4º C) para se a eliminação de restos celulares, e o sobrenadante diluído 1 / 1 (v/v) em Tris-base pH 100 mm 6.8, EDTA 4mM, SDS 8%, glicerol 16%. O teor protéico foi estimado através do método descrito por Peterson (1977) e a concentração protéica estimada a partir de uma curva padrão de soro albumina bovina. Para a comparação dos sinais obtidos, a mesma quantidade proteíca (70 mg por poço) para cada amostra foi eletrotransferida ao minigel de SDS-PAGE 10% (após a adição de bromofenol 0,2% azul e β-mercaptoetanol 8%) e transferidos para as membranas de nitro-celulose ou polivinilideno de fluoreto (PVDF). Para verificar a eficiência do processo de transferência, os géis foram corados com Coomassie blue (azul Coomassie R-250 0,1%, metanol 50%, ácido acético 7%) e as membranas com Ponceau 0,5% em ácido acético 1%.

Após este processo, os blots foram incubados em solução de bloqueio (5% leite em pó desnatado em tampão Tris-salina TBS) durante 1 h em temperatura ambiente e depois incubados a 4 º C com anti-p-Akt (Ser^{473}) (Sigma Chemical Co., 1:1000), anti-p-CREB (Ser^{133}) (Cell Signaling, 1:1000), anti-p-GSK-3β (Ser^{9}) (Cell Signaling, 1:1000), anti-p-ERK1/2 (Cell Signaling, 1:2000), anti-ERK1/2 (Sigma Chemical, 1:40000), anti-Akt (Sigma Chemical, 1:1000), anti-GSK-3β (Cell Signaling, 1:1000), anti-CREB (Cell Signaling, 1:1000) e anti-BDNF (Millipore, 1:1000), todos em TBS com 0,05% de Tween 20 (TBS-T). Em seguida, as membranas foram incubadas com anticorpo anti-coelho conjugado com peroxidase (HRP) (1:5.000) por 1 h e a imunorreatividade das

bandas foi desenvolvida por quimioluminescência (LumiGLO®, Cell Signaling, Beverly, MA, EUA). Todas as etapas de bloqueio e incubação foram seguidas por três lavagens (5 min) das membranas com TBS-T. A fim de se detectarem as formas fosforiladas e total de ERK1/2, Akt e de CREB na mesma membrana, os imunocomplexos foram removidos conforme descrito previamente por Posser et al. (2007). Resumidamente, as membranas foram lavadas uma vez com água duplamente deonizada (5 min), incubadas com NaOH 0,2 M (5 min), lavadas com água duplamente deonizada (5 min) e com TBST (10 min). As membranas desprovidas de imunocomplexos foram bloqueadas e submetidas ao mesmo protocolo descrito acima. A fim de se assegurar que a mesma quantidade proteíca foi adicionada em cada grupo experimental, a quantidade de β-actina foi avaliada usando um anticorpo de rato anti-β-actina (Santa Cruz, 1:1000) e anti-rato HRP-conjugado (Millipore 1 : 4000) como um anticorpo secundário.

A densidade óptica (DO) das bandas foi quantificada usando Scion Image software®. O nível de fosforilação da Akt, CREB, GSK-3β e ERK1/2 foram determinados pela razão entre a DO fosforilada /DO total. O imunoconteúdo de BDNF foi determinado pela relação entre a DO de BDNF / DO de β -actina.

Um diagrama de todo o cronograma experimental está ilustrado na **Figura 6**.

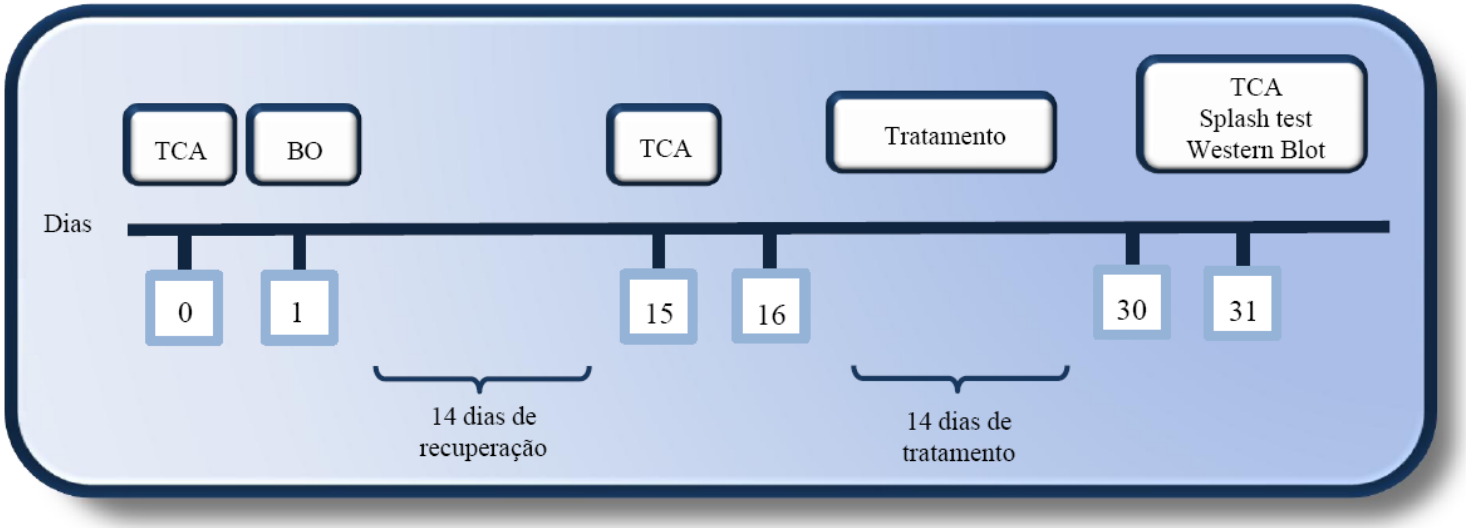

Figura 6. Diagrama de todo o cronograma experimental. O efeito da BO sobre atividade locomotora foi avaliado no TCA em 3 diferentes momentos: pré-cirúrgico, 2 semanas pós cirurgia e 2 semanas após o tratamento. Os animais passaram por um período de recuperação de 14 dias antes do início dos tratamentos. Extrato de *Tabebuia avelanedae* (10-30 mg/kg, p.o.) e fluoxetina (10 mg/kg, p.o.) foram administrados diariamente por via oral (gavagem) durante 14 dias. 24 h após os últimos tratamentos, os animais foram submetidos ao TCA, splash teste, os cérebros rapidamente dessecados e os hipocampos removidos e preparados para o western blot.

4.6. Análise estatística

Os resultados do modelo da bulbectomia olfatória foram avaliados por análise de variância (ANOVA), de uma ou duas vias (de acordo com o protocolo experimental), seguido pelo *post-hoc* de Duncan's quando apropriado. Possíveis relações entre os efeitos comportamentais e a modulação de vias de sinalização foram avaliadas através de correlação de Pearson.

Os dados da investigaçao da participação do sistema glutamatérgico (receptores NMDA) e da via da L-arginina-NO/GMPc foram avaliados por análise de variância (ANOVA), de duas vias seguido pelo *post-hoc* de Tukey's HSD, quando apropriado.

Valores de $P < 0,05$ foram considerados significativos.

5. Resultados

O presente trabalho resultou na confecção de três manuscritos listados abaixo:

Manuscrito 1: "Involvement of GSK-3β, ERK, CREB and BDNF on the antidepressant-like action of the bark ethanolic extract from *Tabebuia avellanedae* in the olfactory bulbectomy model in mice" (Em fase final de preparação).

Manuscrito 2: "Fluoxetine modulates hippocampal cell signaling pathways implicated in neuroplasticity and abolishes depressive-related behavior induced by olfactory bulbectomy in mice" (Submetido à revista Behavioural Brain Research).

Manuscrito 3: "Participation of NMDA receptors and L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like action of the ethanolic extract from *Tabebuia avellanedae* in mice" (Em fase final de preparação).

5.1. Manuscrito 1

Involvement of GSK-3β, ERK, CREB and BDNF on the antidepressant-like action of the bark ethanolic extract from *Tabebuia avellanedae* in the olfactory bulbectomy model in mice

[Running Head: *T. avellanedae* effect on GSK-3β, ERK, CREB and BDNF in the OB model]

Andiara E Freitas[a], Daniele G Machado[a], Josiane Budni[a], Vivian B Neis[a], Grasiela O Balen[a], Mark W Lopes[a], Luiz F de Souza[a], Patricia O Veronezi[b], Melina Heller[b], Gustavo A Micke[b], Moacir G Pizzolatti[b], Alcir L Dafre[a], Rodrigo B Leal[a], Ana Lúcia S. Rodrigues[a,*]

[a]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário – Trindade - 88040-900, Florianópolis-SC, Brazil

[b]Departamento de Química, Centro de Ciências Físicas e Matemáticas, Universidade Federal de Santa Catarina, Campus Universitário – Trindade - 88040-900, Florianópolis-SC, Brazil

* Corresponding author. Tel.: +55 (48) 3721-5043; fax: +55 (48) 3721-9672.

E-mail address: analucia@mbox1.ufsc.br or ana.rodrigues@pq.cnpq.br

Abstract: *Tabebuia avellanedae* Lorentz ex Griseb is a plant employed in tropical America folk medicine for the treatment of depressive symptoms. In this work we investigated the ability of the repeated (14 days) p.o. administration of the bark extract from *T. avellanedae* to: a) cause an antidepressant-like effect in the tail suspension test (TST); b) alter hippocampal Akt, GSK-3β, ERK1/2, CREB phosphorylation and BDNF immunocontent; c) reverse behavioral (hyperactivity and anhedonic behavior) and biochemical (hippocampal Akt, GSK-3β, ERK1/2, CREB, phosphorylation and BDNF immunocontent) changes induced by olfactory bulbectomy (OB), a model of depression, in mice. Extract increased both CREB (Ser^{133}) and GSK-3β (Ser^{9}) phosphorylation (at dose of 10-30 and 30 mg/kg, respectively). OB caused an increased CREB and ERK1 phosphorylation and BDNF immunocontent. The extract prevented the OB-induced hyperactivity, anhedonia (loss of motivational and self care behavior), increase in ERK1 phosphorylation and BDNF immunocontent. Akt (Ser^{473}) and ERK2 phosphorylation was not altered in any group. In conclusion, the antidepressant-like effect elicited by the repeated administration of the extract may be associated with CREB and GSK-3β phosphorylation. Additionally, the extract ability to prevent the behavioral changes induced by OB appears to be mediated by ERK1 and BDNF neuroprotection pathways. Noteworthy, our results indicate that this plant could constitutive an attractive strategy for the management of agitated depression and/or depressive disorder associated with anhedonia.

Keywords: antidepressant; olfactory bulbectomy, open-field; splash test; *Tabebuia avellanedae*; tail suspension test; GSK-3β; ERK1/2; CREB; BDNF.

1. Introduction

Depression is a chronic, recurring and potentially life-threatening illness that affects up to 17% of the population across the globe (Kessler et al., 2005). It is one of the top ten causes of morbidity and mortality worldwide based on projections from the World Health Organization (Murray et al., 1997). Depressive symptoms includes somatic and cognitive alterations such as: depressed mood, anhedonia (loss of interest or pleasure in almost all activities), irritability, feelings of hopelessness, worthlessness or guilt, decreased ability to concentrate and think, decreased or increased appetite, weight loss or weight gain, insomnia or hypersomnia, psychomotor retardation or agitation, fatigue, low energy and recurrent thoughts of death and suicide. A diagnosis of major depression is made when at least five of these nine symptoms are reported for longer than a 2 week period of time, and requires the presence of at least 1 cognitive symptom (depressed mood or anhedonia) present in most of the time (American Psychiatry Association, 2000). The major pharmacologic treatment strategies for depression in clinic practice include tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs) (Kiss, 2008).

Antidepressant drugs increase the rate of adult hippocampal proliferation after chronic but not acute treatment (Sairanen et al., 2005). This idea corroborates with the neurotrophin hypothesis of depression, which invokes increased expression of brain-derived-neurotrophic factor (BDNF), resulting from augmented neural activity elicited by antidepressant medications (Russo-Neustadt and Chen, 2005). BDNF acts through a complex array of intracellular signaling pathways (Tardito et al., 2006), such as the phosphatidylinositol 3′-kinase (PI-3K)-Akt pathway (Numakawa et al., 2010) and the mitogen-activated protein kinase (MAPK) pathway (Chen et al., 2007). Several MAPK

cascades have been characterized, of which the best studied MAPKs are the extracellular signal-regulated kinases ERK1 and ERK2. These pathways, as well as several others that promote neuronal survival, converge on a transcriptional regulator, cyclic-AMP responsive-element binding protein (CREB). CREB-dependent transcription is activated when CREB is phosphorylated on Ser^{133} by a number of protein kinases (Tardito et al., 2006). A link between CREB and BDNF is strongly suggested by the finding that antidepressant-mediated up-regulation of BDNF is blocked in CREB-deficient mice (Conti et al., 2002).

Additionally, several lines of evidence have shown that depressive disorders are related to the activation of the enzyme glycogen synthase kinase-3β (GSK-3β), through a failure on its mechanism of inhibition mediated by phosphorylation (Beaulieu et al., 2009). Kinases such as Akt/protein kinase B (PKB), protein kinase A (PKA) and protein kinase C (PKC) regulate negatively the activity of GSK-3β by phosphorylation at the N-terminal serine 9 (Ser^9) (Beaulieu et al., 2009). GSK-3β is a multifunctional and highly active serine/threonine kinase that was named after its involvement in glycogen metabolism (Jope and Roh, 2006). GSK-3 (isoforms α and β) is an important regulator of glycogen synthesis, gene transcription, synaptic plasticity, apoptosis (cell death), cellular structure and resilience (Jope, 2003). It is well-established that GSK-3 regulates behavior by affecting β-catenin, glutamate receptors, circadian rhythms, and serotonergic neurotransmission (Beaulieu et al., 2008). All of these have been implicated in the pathophysiology of severe mood disorders and studies have shown that GSK-3β inhibitors could constitute new antidepressant drugs (Rosa et al., 2008; Du et al., 2010).

Although the current pharmacotherapy of depression includes a battery of drugs, many are inconsistently effective and exert undesirable side effects (Morilak and Frazer,

2007). Therefore, considerable efforts are invested in development of better drugs and even combined treatments approaches for depression management.

Tabebuia avellanedae Lorentz ex Griseb (Bignoniaceae) is a tree native to tropical rain forests in the northeast of Brazil commonly known as “pau d'arco” or “ipê-roxo”. Recently Freitas et al. (2010) demonstrated that the acute administration of the ethanolic extract from barks of *T. avellanedae* exerts an antidepressant-like effect in the tail suspension test (TST), a behavioral test used to assess the efficacy of antidepressant compounds (Steru et al., 1985). Additionally, the antidepressant-like action of this extract was reported to be mediated by an activation of the monoaminergic systems. Furthermore, the extract from *T. avellanedae* produced a synergistic antidepressant-like effect when combined with conventional antidepressants (Freitas et al., 2010). The TST is a behavioral test used to assess antidepressant activity of different classes of compounds (Steru et al., 1985; Cryan et al., 2005a). However, a major limitation of this model is that antidepressant activity is detected following acute administration, whereas in clinical depression, several days or weeks elapse before a therapeutic effect is observed (Nestler et al., 2002). A widely used animal model of depression is olfactory bulbectomy (OB) , a model able to detect antidepressant activity almost exclusively following chronic antidepressant treatment (Leonard, 1984). OB has been proposed as an animal model of depression in terms of construct validity, since it induces alterations in behavior, and in the endocrine, immune and neurotransmitter systems that reproduces many of those seen in patients with major depression (Kelly et al., 1997; Song and Leonard, 2005).

Considering the antidepressant potential of *Tabebuia avellanedae*, the aims of the present study was to investigate the ability of the repeated (14 days) p.o. administration of the extract from *T. avellanedae* to: a) alter hippocampal Akt, GSK-3β,

ERK1/2 and CREB phosphorylation and BDNF immunocontent; c) reverse behavioral (hyperactivity and anhedonic behavior) and biochemical (hippocampal Akt, GSK-3β, ERK1/2 and CREB phosphorylation and BDNF immunocontent) changes induced by olfactory bulbectomy (OB) in mice. In addition, considering that OB influence on cellular signaling pathways is not well established, this study was aimed to verify the effect of OB procedure alone on these signaling targets associated with neuronal survival/death.

2. Methods and Materials

2.1. Plant material and preparation of the ethanolic extract from T. avellanedae

T. avellanedae barks were provided by Chamel Indústria e Comércio de Produtos Naturais Ltda (Campo Largo, Brazil), lot 4753. The identification was performed by botanist Elide Pereira dos Santos and a voucher specimen has been deposited at the Herbarium of the Department of Botany at the Universidade Federal do Paraná (UFPR), Brazil. Dried and powdered barks (5 kg) were extracted three times by maceration with 95% ethanol for 7 days at room temperature. The combined ethanolic extract was filtered, the solvent evaporated under reduced pressure (40-50°C) and lyophilized to give a red-brown solid (919.2 g).

2.2. Phytochemical analyses of the bark ethanolic extract from T. avellanedae

The analyses of the bark ethanolic extract from *Tabebuia avellanedae* was performed in a capillary electrophoresis system (CE) (HP3DCE, Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector set a 200 nm. The measurements were conducted at 25 °C in an uncoated fused-silica capillary (48.5 cm×50 μm I.D.×375 μm O.D.) obtained from Polymicro (Phoenix, AZ, USA). In the

first conditioning, the capillary was washed for 30 min with sodium hydroxide 1.0 M followed deionized water for 30 min. Between runs the capillary was rinsed for 5 min with running electrolyte (sodium tetraborate 20 mmol L-1 and methanol 10%, pH 9.0) Standard solutions and samples were introduced from the inlet capillary extremity and injected hydrodynamically at 50 mbar (50 mbar=4996.2 Pa) for 6 s. The applied separation voltage was 30 kV, positive polarity in the injection side. Caffeic acid (100 mg L-1 was utilized as internal standard and detection at 330 nm. Data acquisition and treatment were performed with HP Chemstation software.

Sample preparation: 0,5299 g of the ethanolic extract was solubilized into 10 mL of the methanol:water 50% (v/v).

2.3. Animals

Female Swiss mice (2 month old, 30-40g) were maintained at constant room temperature (20-22°C) with free access to water and food, under a 12:12h light:dark cycle (lights on at 07:00 h). The cages were placed in the experimental room 24 h before the test for acclimatization. All manipulations were carried out between 9:00 and 17:00 h, with each animal used only once. The procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee. All efforts were made to minimize animal suffering and the number of animals used in the experiments.

2.4. Surgical procedure

After a 2-week acclimatization period, the bilateral OB was performed by suction method described previously by Leonard and Tuite (1981). Animals were randomly divided into two groups: OB and sham-operated animals. Briefly, mice were anesthetized with xylazine (6 mg/kg, i.p.) in combination with ketamine (100 mg/kg,

i.p.) diluted in saline. An incision was made in the skin overlying the skull, and, after exposure of the skull, holes were drilled on both sides of the mid-line. Then the olfactory bulbs were bilaterally aspired bilaterally aspirated by blunt hypodermic needle (with for 1.0 to 1.2 cm long and with a rounded tip of 0.80 to 1.2 mm of diameter) attached the syringe (10 ml) for the suction, taking care not to cause damage to the frontal cortex. The holes were filled with swab in order to stop the bleeding and covered with dental cement. All surgical procedure was carried out employing alcohol 70% to eliminate contaminations. Sham-operated animals were treated in the same way except the bulbs were left untouched. Mice were allowed to recover under warming help with body temperature maintenance. The animals were given 14 days to recover following surgery prior to treatments.

2.5. Drugs and treatment

The extract from *T. avellanedae* (10-100 mg/kg) was dissolved in distilled water with 5% Tween 80 and was administered by oral route (p.o.) by gavage. The dissolution of the extract was freshly done from the liophylized power immediately before its administration. A control group received distilled water with 5% Tween 80 as vehicle.

In the experiments designed to study the antidepressant-like effect of the repeated treatment (for 14 days) of the extract, the immobility time in the TST and the locomotor activity in the open-field were assessed in independent groups of mice 24 h after the last daily administration of the extract (10-100 mg/kg, p.o.). This experiment was carried out in order to select the doses of the extract to be used to reverse the depressive-like behavior of olfactory bulbectomized mice.

Two weeks after surgery, the extract (10-30 mg/kg, p.o.) was administered once daily for 14 days. Animals were assigned to the following groups: (a) sham/vehicle, (b) sham/extract 10 mg/kg and (c) sham/extract 30 mg/kg, as the control group; (d)

OB/vehicle, (e) OB/extract 10 mg/kg and (f) OB/extract 30 mg/kg. Number of mice per group was 9-12.

2.6. Open-field test

The OB model of depression reproduces in preclinical assays the psychomotor agitation consistent with that is observed in agitated depression (Zueger et al., 2005). To assess the effects of the OB on locomotor activity, mice were evaluated in the open-field paradigm as previously described (Rodrigues et al., 1996). The test was consecutively performed in 3 time periods: pre-surgically, after 2 weeks of surgery, and 2 weeks post-treatment. The number of squares crossed with all paws (crossing) and was counted in a 6 min session. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.7. Tail suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al. (1985). Mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was registered 2 weeks post-treatment during a 6-min period (Machado et al., 2009; Freitas et al., 2010).

2.8. Splash test

The splash test was carried out 24 h after the last repeated drug administration as described by Isingrini and co-workers (2010), with minor modifications. The test consists of squirting a 10% sucrose solution on the dorsal coat of a mouse placed individually in clear Plexiglas boxes (9 x 7 x 11 cm). Because of its viscosity, the sucrose solution dirties the mouse fur and animals initiate grooming behavior. After

applying sucrose solution, the latency to grooming and the grooming time were manually recorded for a period of 5 minutes as an index of self-care and motivational behavior, considered to be parallel with some symptoms of depression such as apathetic behavior (Willner 2005; Yalcin et al., 2005). The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.9. Western Blot

After two weeks of treatment, and 24 h after the last administration of extract or vehicle by oral rote, mice were decapitated. Brains were removed and the lesion was estimated macroscopically immediately after brain removal; all brains with incomplete surgery or cortex damage were discarded from the experiment. The hippocampus was rapidly dissected and placed in cold saline solution. Western blot analysis was performed as previously described (Leal et al., 2002; Cordova et al., 2004). Briefly, hippocampal tissue were mechanically homogenized in 400 µl of Tris-base 50 mM pH 7.0, EDTA 1 mM, sodium fluoride 100 mM, PMSF 0.1 mM, sodium vanadate Na_3VO_4 2 mM, Triton X-100 1%, glycerol 10%, and then incubated for 30 min in ice. Lysates were centrifuged (10000 x g for 10 min, at 4ºC) to eliminate cellular debris, and supernatants diluted 1/1 (v/v) in Tris-base 100 mM pH 6.8, EDTA 4 mM, SDS 8%, glycerol 16%. Protein content was estimated with the method described in Peterson (1977) and concentration calculated by a standard curve with bovine serum albumin. To compare signals obtained, the same amount of protein (70 µg per lane) for each sample was electrophoresed in 10% SDS-PAGE minigels (after addition of bromophenol blue 0.2% and β-mercaptoethanol 8%) and transferred to nitro-cellulose or polyvinylidene fluoride (PVDF) membranes. To verify transfer efficiency process, gels were stained

with Coomassie blue (Coomassie blue R-250 0.1%, methanol 50%, acetic acid 7%) and membranes with Ponceau 0.5% in acetic acid 1%.

After this process, blots were incubated in a blocking solution (5% non-fat dry milk in Tris buffer saline solution, TBS) for 1 h at room temperature and then probed at 4ºC with anti-phospho-Akt (Sigma Chemical Co., 1:1000), anti-phospho-CREB (Ser^{133}) (Cell Signaling, 1:1000), anti-phospho-GSK-3β (Cell Signaling, 1:1000), anti-phospho-ERK1/2 (Cell Signaling, 1:2000), anti-ERK1/2 (Sigma Chemical, 1:40000), anti-Akt (Sigma Chemical, 1:1000), anti-GSK-3β (Cell Signaling, 1:1000), anti-CREB (Cell Signaling, 1:1000) and anti-BDNF (Millipore, 1:1000), all in TBS containing 0.05% Tween 20 (TBS-T). Next, the membranes were incubated with anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody (1:5,000) for 1 h and the immunoreactive bands were developed by chemiluminescence (LumiGLO®, Cell Signaling, Beverly, MA, USA). All blocking and incubation steps were followed by three washes (5 min) of the membranes with TBS-T. In order to detect phosphorylated and total forms of ERK1/2, Akt and CREB in the same membrane, the immunecomplexes were stripped as previously described (Posser et al., 2007). Briefly, membranes were washed once with double deonized water (5 min), followed by incubation with NaOH 0.2 M (5 min), washing with double deonized water (5 min) and with TBST (10 min). The membranes stripped of immune complexes were blocked and followed the same steps decribed above. In order to ascertain the same protein load for each experimental group the house keeping protein, β-actin, was evaluated using a mouse anti-β-actin antibody (Santa Cruz, 1:1000) and anti-mouse HRP-conjugated (Millipore 1:4000) as a secondary antibody.

The optical density (O.D.) of the bands was quantified using Scion Image software®. The phosphorylation level of Akt, CREB, GSK-3β and ERK1/2 were

determined as a ratio of O.D. of phosphorylated band/O.D. of total band. The immunocontent of BDNF were determined making the relationship between the O.D of BDNF band/O.D of β actin band.

A diagram of all experimental schedule is given in Fig. 1.

2.9. Statistical analysis

Comparisons between experimental and control groups (except Western Blot assays) were performed by one-way or two-way ANOVA followed by Duncan's HSD test when appropriate. $P<0.05$ was considered significant.

3. Results and Discussion

3.1. Phytochemical analyses of the ethanolic extract from T. avellanedae

Fig. 2 shows the electropherogram of a sample of the bark ethanolic extract from *T. avellanedae*. The analysis of the electropherogram, which has signals at migration time in the range of 2,1-6,5 min, allowed us to identify p-hydroxybenzoic acid (1), anisic acid (2), veratric acid (3) and caffeic acid (4).

3.2. Effect of the repeated (14 days) administration of the ethanolic extract from T. avellanedae on the immobility time in the TST and locomotor activity in the open-field test

The results depicted in Fig. 3A show that the ethanolic extract from *T. avellanedae* given by oral route for 14 days decreased the immobility time in the TST, a behavioral profile characteristic of an antidepressant-like effect. One-way ANOVA revealed a significant effect of the extract [$F(3,29)=4.42$, $P<0.01$]. Post hoc analysis

indicated a significant decrease in the immobility time elicited by the repeated administration of the extract at doses of 10 and 30 mg/kg (p.o.). Fig. 3B shows that the administration of extract during 14 days (dose range 10-100 mg/kg, p.o.) produced no effect in the locomotor activity assessed in the open-field test [$F(3,30)=0.10$, $P=0.96$].

3.3. Effect of the OB on locomotor activity in the open-field test

Fig. 4 shows that OB procedure increased mice ambulation in the OB-mice group as compared with the same group at pre-operative period. Two-way ANOVA revealed a significant effect of OB [$F(1,180)=33.55$, $P<0.01$], period [$F(1,180)=7.11$, $P<0.01$] and OB x period interaction [$F(1,180)=47.58$, $P<0.01$]. Post hoc analyses indicated a significant augmentation in locomotor activity elicited by OB.

3.4. Effect of the repeated (14 days) treatment with the extract from T. avellanedae on hyperactivity induced by OB

The results presented in Fig. 5 show that the repeated administration of the extract (10 and 30 mg/kg, p.o.) was able to reverse the hyperactivity induced by OB in the open-field apparatus. A two-way ANOVA revealed significant differences of OB [$F(1,52)=68.87$, $P<0.01$], treatment [$F(2,52)=10.74$, $P<0.01$] and OB x treatment interaction [$F(2,52)=4.49$, $P=0<0.1$]. Post hoc analyses indicated that the extract (10 and 30 mg/kg, p.o.) treatment for 14 days to OB-mice prevented the hyperactivity caused by OB.

3.5. Effect of the extract from T. avellanedae administration (14 days) on anhedonic behavior induced by OB

The results depicted in Fig. 6A illustrates that the increase in latency to grooming, an indicative of anhedonic behavior, produced by OB was significantly blocked by extract (10 and 30 mg/kg, p.o.) treatment during 14 days. The two-way ANOVA revealed significant differences of OB [$F(1,51)=7.99$, $P<0.01$], treatment [$F(2,51)=4.04$, $P<0.05$] and OB x treatment interaction [$F(2,51)=4.52$, $P<0.01$]. Also, Fig. 6B shows that the reduced grooming time caused by OB, another parameter used to infer anhedonic behavior, was significantly reversed by the extract. A two-way ANOVA revealed significant differences of OB [$F(1,54)=24.02$, $P<0.01$], treatment [$F(2,54)=3.99$, $P<0.05$] and OB x treatment interaction [$F(2,54)=15.93$, $P<0.01$]. Post hoc analyses indicated that the extract (10 and 30 mg/kg, p.o.) treatment (for 14 days) was able to prevent the anhedonic behavior (expressed by the increase in latency to grooming and the decreased grooming time) in the splash test caused by olfactory bulbs ablation.

3.6. Evaluation of Akt, GSK-3β, ERK1/2 and CREB phosphorylation and BDNF immunocontent by Western blot assay

Western blot analysis from hippocampal tissue homogenates showed that neither the OB procedure nor the repeated treatment with the extract from *T. avellanedae* altered Akt phosphorylation in hippocampus of mice (Fig. 7B). The two-way ANOVA revealed no differences for OB [$F(1,18)=0.31$, $P=0.58$], treatment [$F(2,18)=0.14$, $P=0.86$] and OB x treatment interaction [$F(2,18)=0.28$, $P=0.76$]. Fig. 7D illustrates the effect of the treatment (14 days) of OB-mice with the extract on GSK-3β phosphorylation. The two-way ANOVA revealed a significant main effect of treatment

[F(2,18)=7.52, P<0.01], but did not show a significant effect of OB [F(1,18)=3.35, P=0.08] and OB x treatment interaction [F(2,18)=0.002, P=0.99]. Post hoc analyses indicated that OB produced no effect on the GSK-3β phosphorylation (Ser^9) as compared with Sham-operated group. Furthermore, the treatment (14 days) of Sham or OB-mice with extract (30 mg/kg, p.o.) caused a significant increase in GSK-3β phosphorylation as compared with Sham-operated group or OB-vehicle group, respectively.

The results depicted in Fig. 8B illustrates that the increase in ERK1 phosphorylation produced by OB was significantly prevented by extract (10 and 30 mg/kg, p.o.) treatment during 14 days. The two-way ANOVA revealed a significant main effect of OB [F(1,18)=5.44, P<0.05], and OB x treatment interaction [F(2,18)=4.56, P<0.05], but did not show a significant main effect of treatment [F(2,18)=0.94, P=0.40]. Post hoc analyses indicated that the extract (10 and 30 mg/kg, p.o.) was able to prevent the increase in ERK1 phosphorylation produced by OB . Fig. 8C illustrates that ERK2 phosphorylation was not altered in any experimental condition. The two-way ANOVA revealed no differences for OB [F(1,18)=2.25, P=0.15], treatment [F(2,18)=0.81, P=0.46], and OB x treatment interaction [F(2,18)=1.23, P=0.32].

Finally, the effect of the repeated treatment of OB-mice with extract on CREB phosphorylation and BDNF immunocontent were verified by western blot assay. Fig. 9B shows that the extract administration for 14 days and the OB procedure caused a significant increase in CREB phosphorylation. The two-way ANOVA revealed a significant main effect of OB [F(1,14)=6.78, P<0.05] and treatment [F(2,14)=3.50, P<0.05], but did not show a significant effect of OB x treatment interaction

[F(2,14)=2.59, P=0.11]. Post hoc analyses indicated that the OB surgery and the repeated treatment with extract (10 and 30 mg/kg, p.o.) were able to increase CREB phosphorylation, as compared with Sham-operated group and vehicle-treated group, respectively. Fig. 9D shows that the repeated treatment of OB-mice with extract was able to prevent the BDNF immunocontent augmentation caused by olfactory bulbs ablation. The two-way ANOVA revealed a significant main effect of OB [F(1,18)=7.68, P<0.01] and OB x treatment interaction [F(2,18)=3.93, P<0.05], but did not show a significant main effect of extract treatment [F(2,18)=0.54, P=0.59]. Post hoc analyses indicated that OB-induced increase in BDNF immunocontent was blocked by treatment (14 days) of OB-animals with extract at doses of 10 and 30 mg/kg (p.o.).

This study supports pharmacological and biochemical evidence for the antidepressant-like effect of the extract from *T. avellanedae* which was recently demonstrated, by our group, to be dependent on its interaction with the monoaminergic systems (Freitas et al., 2010). In the present work, we demonstrated that the extract from *T. avellanedae* administered by oral route during 14 days produced a significant antidepressant-like effect in the TST, a commonly used behavioral test that predict the efficacy of antidepressant treatment (Bourin et al., 2005). Additionally, a consistent antidepressant-like activity of the extract from *T. avellanedae* in a well-established animal model of depression – the olfactory bulbectomy model was shown. The OB model of depression standardized in our laboratory was able to reproduce several behavioral changes reported in literature, mainly the increased locomotor activity in the open-field paradigm and the anhedonic behavior in the splash test, providing validation for the model. The treatment for 14 days with the extract from *T. avellanedae* in bulbectomized mice was effective in preventing the hyperactivity and the anhedonic behavior induced by olfactory bulbs ablation. In addition, significantly extending data

regarding OB influence on the expression of proteins implicated in cellular survival/death, this study investigated by western blot analysis the effects of OB and/or extract on Akt, GSK-3β, ERK1/2, CREB and BDNF. OB caused a significant increase in CREB and ERK1 phosphorylation and BDNF immunocontent. The extract per se caused an augmentation of both CREB and GSK-3β phosphorylation. In addition, it was able to reverse the increase in ERK1 phosphorylation and BDNF immunocontent induced by OB. Altogether, the results suggest that the antidepressant-like action of the extract from *T. avellanedae* appears to be dependent on the modulation of GSK-3β, ERK1, CREB and BDNF-mediated signaling pathways.

The TST is a predictive animal test widely used for screening antidepressant activity of different classes of drugs (Porsolt et al., 1977; Cryan et al., 2005a). This test is based on the observation that animals, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable stressful situation. When antidepressant treatments are given prior to the tests, the subjects will actively persist engaging in escape-directed behavior for longer periods of time than after vehicle treatment (Cryan et al., 2005a). Recently, our group showed that an acute administration of the extract from *T. avellanedae* produced an antidepressant-like effect in the TST in mice (Freitas et al., 2010). In the present study, the treatment of mice for 14 days with extract at dose of 10 and 30 mg/kg (p.o.) produced a significant antidepressant-like effect in the TST in agreement with the fact that antidepressant drugs produce a reduction in the immobility time in this predictive test (Artaiz et al., 2005). This experiment show that no tolerance was verified following repeated treatment of the extract at doses of 10 and 30 mg/kg (p.o.), since the extract administered acutely causes an antidepressant-like effect in the TST at the doses of 10, 30, 100 and 300 mg/kg (p.o.) (Freitas et al., 2010).

Besides TST, there are several animal models to test antidepressant efficacy, such as the FST (Cryan et al., 2005b), and the learned helplessness (Seligman et al., 1975). A major drawback with these models is that antidepressant activity is detected following acute administration, whereas in clinical depression, several days or weeks are required before a therapeutic effect is observed (Yan et al., 2010). OB is capable of detecting antidepressant activity almost exclusively following chronic drug treatment, and results in behavioral changes reminiscent of various symptoms in depression including learning and memory dysfunctions, psychomotor agitation, altered avoidance behavior and anhedonia (Kelly et al., 1997). The mechanisms underlying the development of depression in OB animals are not well known. However, these effects do not appear to simply be the result of anosmia, as selective ablation of the olfactory sensory receptors does not produce the characteristic symptoms of OB (Alberts and Friedman, 1972; Saitoh et al., 2006).

Psychomotor agitation is one of key-symptoms to diagnosis of agitated depression (American Psychiatry Association, 2000). This symptom is evaluated in the OB model through the evaluation of the OB-mice locomotion in the open-field paradigm and is one parameter that provides validation for the surgery procedure (Kelly et al., 1997). It is well established that classical antidepressants such as fluoxetine (Mar et al., 2002; Roche et al., 2007), amitriptyline (Jarosik et al., 2007) and venlafaxine (McGrath and Norman, 1998) attenuates OB-induced hyperactivity in the open-field apparatus. In the present work, the olfactory bulbs destruction produced a significant hyperlocomotion consistent with clinical symptoms of agitated depression that was prevented by the treatment (14 days) of OB-mice with extract at doses of 10 and 30 mg/kg (p.o.). The ability of the extract to block symptoms of agitated depression reinforces the antidepressant-like property of this plant and corroborates with several

studies that have shown that antidepressant compounds are able to prevent the hyperactivity induced by OB (Nowak et al., 2003; Xu et al., 2005; Breuer et al., 2009).

In the present study, anhedonia, an affective symptom related to the inability to experience pleasure was assessed through the splash test. In this test, an increase in the latency to grooming as well as a decrease in the total grooming time indicates a depressive behavior (Kalueff and Tuohimaa, 2004). Additionally, a reduction in the grooming time in the splash test is considered to parallel the motivational and apathetic behavior observed in depression (Willner, 2005). Recent studies have shown that antidepressant compounds are effective in preventing anhedonia in animal models of depression such as the immobilization stress (Hayase, 2011), chronic unpredictable stress (Li et al., 2011) and chronic mild stress (Elizalde et al., 2010). Our results corroborate with these findings by showing that the destruction of the olfactory bulbs induced anhedonia and a loss of motivational and self care behavior evidenced by the increase in the latency to grooming and the decrease in the grooming time exhibited by the OB-mice group, as compared with the Sham-operated group. Noteworthy, treatment with extract from *T. avellanedae* (10 and 30 mg/kg, p.o.) blocked this loss in motivational and self care behavior and suggests that this extract could constitutive an attractive tool to the management of depression associated with anhedonia in clinical practice.

Considering that the efficacy of antidepressant agents cannot be solely explained by their actions on the monoaminergic system, and that molecular adaptations mediated by the activation of signaling pathways may underlie their therapeutic action (Fisar and Hroudová, 2010), this study also evaluated the involvement of the Akt, GSK-3β, ERK-1/2, CREB and BDNF-mediated signaling pathways in the hippocampus in the

depressive-like effect produced by OB (thirty days after surgery) and the ability of the repeated treatment with the extract from *T. avellanedae* to alter these parameters in OB and Sham-operated mice.

Akt/PKB protein kinase, a serine/threonine kinase, belongs to the cAMP-dependent protein kinase A/protein kinase G/ protein kinase C (AGC) super family of protein kinases that share structural homology within their catalytic domain and have the similar mechanism of activation (Song et al., 2005). In mammals, Akt comprises three highly homologous members known as PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3). All Akt/PKB isoforms except the PKBγ/Akt3 splice variant contain two regulatory phosphorylation sites, Thr308 in the activation loop within the kinase domain and Ser^{473} in the C-terminal regulatory domain (Nicholson and Anderson, 2002). PKB/Akt is activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components (Song et al., 2005). PKB/Akt phosphorylates and regulates the function of many cellular proteins involved in processes that include metabolism, apoptosis, and proliferation (Song et al., 2005). In the present work, the repeated treatment with extract (10 and 30 mg/kg, p.o.) did not produce any effect on Akt phosphorylation (Ser^{473}) in the hippocampus. Similarly, a study by Rantamäki et al. (2007) found no evidence of increased or decreased activity of the Akt pathway in the hippocampus after acute or chronic treatment of mice with fluoxetine (30 mg/kg, i.p.). Moreover, OB caused no alteration on Akt phosphorylation (Ser^{473}) in the hippocampus, indicating that the depressive-like behavior of bulbectomized mice seem not to be associated with any impairment on Akt-mediated signaling pathway.

GSK-3 was characterized in 1980 as a protein that phosphorylated and deactivated glycogen synthase (Embi et al., 1980). It is a ubiquitous kinase, found in both neurons and glia, localized to the cytoplasm, nucleus, and mitochondria (Jope and Johnson, 2004). It is generally considered constitutively active and found as two isoforms, α and β. In general, increased activity of GSK-3 is proapoptotic, whereas inhibiting GSK-3 prevents apoptosis. GSK-3β has been suggested to be implicated in the pathogenesis of bipolar disorder, schizophrenia and depression. This enzyme is proposed to be an important target for drugs used in the therapy of these disorders (Beaulieu et al., 2009). Many of the pathways that use GSK-3β as a regulator have links to human diseases. The primary GSK-3β mechanism of regulation involves inhibitory phosphorylation of their N-terminal Ser^9 (Peineau et al., 2008). Several kinases including Akt/PKB, PKA, PKC, and ribosomal S6 kinase phosphorylate the inhibitory site to inhibit GSK-3β activity (Doble and Woodgett, 2003). In contrast, protein phosphatase 1 (PP1) and 2A dephosphorylate the inhibitor site of GSK-3β resulting in the activation of GSK-3β (Peineau et al., 2007). GSK-3β has also recently been identified as a common target for SSRIs, TCAs, and antipsychotics (Li et al., 2004; Beaulieu, 2007; Li et al., 2007). Noteworthy, the novel thiadiazolidinone NP031115, a putative GSK-3β inhibitor, and the well-established GSK-3β inhibitor AR-A014418 were shown to produce antidepressant-like effects in the mouse FST (Rosa et al., 2008). Furthermore, reduction of GSK-3β phosphorylation, resulting in kinase activation, in the prefrontal cortex has been associated with major depressive disorders in a cohort of suicide victims (Karege et al., 2006). Several studies have shown that the antidepressants treatment induces an augmentation of the inhibitory phosphorylation of GSK-3β on Ser^9 in cerebral cortex (Li et al., 2004) and hippocampus (Eom and Jope, 2009). Our results are consistent with this notion, since the treatment (14 days) of

Sham-operated or OB-mice group with extract from *T. avellanedae* (30 mg/kg, p.o.) caused a significant increase in Ser^9 GSK-3β phosphorylation that indicates an inhibition of GSK-3β activity, as compared with Sham-vehicle or OB-vehicle group in the mouse hippocampus. It remains to be established the importance of the inhibition of GSK-3β activity for the mechanisms underlying the antidepressant-like effect of the extract, especially considering that OB caused no significant change in GSK-3β phosphorylation.

Mitogen-activated protein kinases (MAPK) are members of a superfamily of serine/threonine protein kinases extensively distributed throughout the central nervous system that play a crucial role in transducing signals to the nucleus, and thereby regulate the genes involved in wide variety of cellular processes, including cell proliferation, differentiation, apoptosis, and synaptic plasticity (Johnson and Lapadat, 2002; Strnisková et al., 2002). Several MAPK cascades have been characterized, of which the best studied MAPKs are the extracellular signal-regulated kinases ERK1 and ERK2. When activated, the phosphorylation state of the ERK1/2 primarily regulates neuronal growth, differentiation and apoptosis (Stork and Schmitt, 2002). Recently, a growing body of evidence indicates that the ERK1/2 pathway may participate in modulation of depression (Fumagalli et al., 2005; Todorovic et al. 2009). It has been recently reported that stress-induced depressive-like behaviors were correlated with an increase in hippocampal p-ERK1. Chronic treatment with desipramine prevented this depressive-like behavior and p-ERK1 increase (Bravo et al., 2009), suggesting that the blockade of this signaling pathway might represent an antidepressant mechanism. Our results corroborate with this notion, and with several studies that have been shown that ERK1/2 inhibition produced an antidepressant-like effect (Einat et al., 2003; Galeotti and Ghelardini, 2011), since the increase in ERK1 phosphorylation caused by OB

surgery was significantly prevented by the extract from *T. avellanedae* (10 and 30 mg/kg, p.o.). Additionally, in the present study ERK2 phosphorylation was not altered in any experimental group.

A number of growth factors and hormones have been shown to stimulate the expression of cellular genes by inducing the phosphorylation of the nuclear factor CREB at Ser^{133} (Tardito et al., 2006). This phosphorylation promotes the association of CREB with the CREB-binding protein, a co-activator protein that aids in the assembly of an active transcription complex enabling target gene activation (Lu et al., 2003). Originally characterized as a target for PKA-mediated phosphorylation (Thomson et al., 2008), CREB is also recognized by other cellular kinases including Akt/PKB (Song et al., 2005), PKC (Martín et al., 2009), $pp90^{RSK}$, calmodulin kinases II and IV (Shaywitz and Greenberg, 1999), p38MAPK (Kitamura et al., 2002) and PKG (Pilz and Casteel, 2003). Chronic administration of antidepressants increases CREB-mediated neurogenesis in mouse hippocampus (Li et al., 2009).

Data from literature have shown that antidepressant-like compounds mediate over-expression of CREB in the dentate gyrus of the rat hippocampus (Chen et al., 2001; Qi et al., 2008). In the present study, treatment for 14 days with the extract from *T. avellanedae* (10 and 30 mg/kg, p.o.) produced a significant increase in the CREB phosphorylation in the hippocampus both in Sham-operated and bulbectomized mice, in agreement with the idea that CREB is up-regulated by chronic antidepressant treatment, and that increasing CREB levels in rodent models results in antidepressant-like behaviors (Chen et al., 2001; Qi et al., 2008).

Additionally, it has been proposed that CREB is activated not only in response to the pro-growth and pro-survival stimuli but in response to stressful stimuli as well

(Kageyama et al., 2010). In neurons, CREB is phosphorylated under conditions of hypoxia and oxidative stress, suggesting that the activation of a CREB-dependent survival program in response to harmful stimuli might represent a cellular form of defense (Delivoria-Papadopoulos, 2011). Indeed, several pieces of evidence strongly support a role for CREB as a neuroprotectant (Stern et al., 2011). Some recent studies in rats demonstrated that activation of CREB after cerebral ischemia stimulated cell proliferation in the adult dentate gyrus (Zhu et al., 2004). Additionally, Tian et al. (2009) have shown that that both Src- and ERK- dependent proliferations of adult hippocampal progenitor cells are mediated by activation of CREB, and provide evidence that Src/Raf/ERK cascade are involved in neural cell proliferation evoked by ischemia in dentate gyrus. In this study, the olfactory bulbs ablation produced an increase in CREB phosphorylation and corroborates with the notion that CREB could be activated as form of cellular defense.

Several lines of evidence have linked the transcription factor CREB and one of its target genes, BDNF as mediators of the therapeutic responses to antidepressants (Nair and Vaidya, 2006; Duman, 2009). Chronic antidepressant treatment increases BDNF expression in the hippocampus a brain region implicated in the pathophysiology and treatment of depression (Schmidt and Duman, 2007; Marchetti et al., 2010). BDNF activate a variety of signaling cascades, including the PI-3K-Akt, the MAPK, and the cAMP/PKA pathways (Numakawa et al., 2010).

According to the neurotrophic hypotheses of major depression, BDNF deficit, particularly in the hippocampus, may play a major role in the pathogenesis of major depression (Duman et al., 1997). In the present study, our result is in contrast to the original hypothesis regarding BDNF deficit in depression, since we identified a

significant OB-induced up-regulation of BDNF content at hippocampal tissue that was blocked by treatment (14 days) of bulbectomized mice with extract from *T. avellanedae* at dose of 30 mg/kg (p.o.). Nevertheless, elevated BDNF levels have also been reported in animal models of depression and have been thought to be associated with depression/anxiety behaviors (Groves, 2007). A similar study showed that olfactory bulbectomy in mice leads to increased BDNF levels in the hippocampus and frontal cortex as compared with sham-operated animals (Hellweg et al., 2007). In another study, transgenic over-expression of BDNF was observed in mice exhibiting increased anxiety-like behaviors, comparable to that seen in wild-type mice exposed to chronic immobilization stress (Govindarajan et al., 2006). This work showed that the hyperactivity exhibited by OB-mice is associated with an augmentation of BDNF immunocontent and that both effects were prevented by the repeated treatment with the extract (30 mg/kg, p.o.). Accordingly, increased locomotor activity has been shown to increase BDNF expression in rodent brain (Cotman and Berchtold, 2002; Kitamura et al., 2003). Noteworthy, the increase in BDNF content may also result from a compensatory up-regulation of this neurotrophin following OB that interestingly could be prevented by the extract from *T. avellanedae*.

In conclusion, the present study firstly indicates that the administration of *T. avellanedae* during 14 days in mice is able to produce an antidepressant-like effect in the TST that may be associated with CREB (Ser^{133}) and GSK-3β (Ser^{9}) phosphorylation. We also have shown that the olfactory bulbectomy animal model of depression standardized in our laboratory reproduced in mice the hyperactivity in the open-field paradigm and the anhedonic behavior in the splash test, providing validation for the model. Olfactory bulbs ablation caused a significant augmentation in CREB and ERK1 phosphorylation and BDNF immunocontent. Furthermore, the repeated treatment

with *T. avellanedae* was effective in reversing the hyperactivity and the anhedonic behavior induced by OB and these effects appears to be associated with ERK1 and BDNF neuroprotection pathways. Finally, our results provide evidence that the antidepressant-like effect of *T. avellanedae* is dependent, at least in part, on an interaction with signaling pathways related with neuronal survival, supporting the notion that these targets may be critical to the antidepressant action of this plant. Future studies will be necessary to elucidate the contribution of the isolated compounds (p-hydroxybenzoic acid, anisic acid, veratric acid, and caffeic acid) to the antidepressant activity of the extract from *T. avellanedae*.

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Conflict of Interest Statement

The Authors declare that there is no conflict of interest.

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Legend to the figures:

Figure 1- Diagrams of the all experimental schedule. The effect of OB on locomotor activity was accessed in the open-field paradigm in 3 time periods: pre-surgically, after 2 weeks of surgery, and 2 weeks post-treatment. The animals were given 14 days to recover following surgery prior to treatments. Extract at doses of 10 and 30 mg/kg (p.o.) was administered once daily for 14 days. Twenty-four h after the last administration of fluoxetine, mice were submitted to open-field, and 1 h later to the splash test. Immediately after the behavioral test, hippocampi were rapidly dissected and prepared to western blot assay.

Figure 2- Eletrocpherogram of the ethanolic extract from *Tabebuia avellanedae*. p-hydroxybenzoic acid (1), anisic acid (2), veratric acid (3) and caffeic acid (4).

Figure 3- Effect of an oral repeated (14 days) administration with ethanolic extract from *Tabebuia avellanedae* (dose range 10-100 mg/kg) in the TST (panel A) and open-field test (panel B). Each column represents the mean + S.E.M. (n = 7-9). Statistical analysis was performed by one-way ANOVA, followed by the Duncan's test. * $P<0.05$ as compared with the vehicle-treated group (C).

Figure 4- Effect of the OB on locomotor activity in the open-field test in the post-operative period (2 weeks after OB). Each column represents the mean + S.E.M. of 43-45 animals. Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. ## P<0.01 as compared with the control group (Sham) at pre-operative period.

Figure 5- Effect of the repeated (14 days) treatment of bulbectomized mice with the ethanolic extract from *Tabebuia avellanedae* (10-30 mg/kg, p.o.) in the locomotor activity in the open-field test. Each column represents the mean + S.E.M. of 9-12 animals. Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. **P<0.01 as compared with the control group (Sham-vehicle) and ## P<0.01 as compared with the OB-vehicle group.

Figure 6- Effect of the repeated (14 days) treatment of bulbectomized mice with the ethanolic extract from *Tabebuia avellanedae* (dose range 10-30 mg/kg, p.o.) in the latency to grooming (panel A) and grooming time (panel B) in the Splash test. Each column represents the mean + S.E.M. (n = 9-10). Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. **P<0.01 as compared with the control group (Sham-vehicle) and ## P<0.01 as compared with the OB-vehicle group.

Figure 7- Effect of repeated (14 days) treatment of bulbectomized mice with the ethanolic extract from *Tabebuia avellanedae* (dose range 10-30 mg/kg, p.o.) on Akt (panels A and B) and GSK-3β (panels C and D) phosphorylation. Panels A and C show a representative western blot. Quantitative analyses are illustrated in panels B and D The data are expressed as ratio between phosporylated (P-Akt, P-GSK-3β) and total (T-

Akt, T-GSK-3β) form of Akt and GSK-3β. Each column represents the mean + S.E.M. of 4 experiments. Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. *P<0.05 as compared with the control group (Sham-vehicle) and # P<0.05 as compared with the OB-vehicle group. The bands on the top show the Akt and GSK-3β expression on phosphorylated form (upper bands) and total form (lower bands) referent to sham/vehicle (S-Ct), sham/extract 10 mg/kg (S-E10), sham/extract 30 mg/kg (S-E30), OB/vehicle (B-Ct), OB/extract 10 mg/kg (B-E10) and OB/extract 30 mg/kg (B-E30) groups.

Figure 8- Effect of repeated (14 days) treatment of bulbectomized mice with the ethanolic extract from *Tabebuia avellanedae* (dose range 10-30 mg/kg, p.o.) on ERK1 (panels A and B) and ERK2 (panels A and C) phosphorylation. Panel A shows a representative western blot. Quantitative analyses are illustrated in panels B and C. The data are expressed as ratio between phosporylated (P-ERK1 and P-ERK2) and total (T-ERK1 and T-ERK2) forms. Each column represents the mean + S.E.M. of 4 experiments. Statistical analysis was performed by two-way ANOVA, followed by Duncan's test. *P<0.05 and **P<0.01 as compared with the control group (Sham-vehicle) and # P<0.05 as compared with the O.B.-vehicle group. The bands on the top show the ERK1 and ERK2 expression on phosphorylated form (upper bands) and total form (lower bands) referent to sham/vehicle (S-Ct), sham/extract 10 mg/kg (S-E10), sham/extract 30 mg/kg (S-E30), OB/vehicle (B-Ct), OB/extract 10 mg/kg (B-E10) and OB/extract 30 mg/kg (B-E30) groups.

Figure 9- Effect of repeated (14 days) treatment of bulbectomized mice with the ethanolic extract from *Tabebuia avellanedae* (dose range 10-30 mg/kg, p.o.) on CREB

(panels A and B) phosphorylation and BDNF (panels C and D) immunocontent. Panels A and C show a representative western blot. Quantitative analyses are illustrated in panels B and D. The data are expressed as ratio between phosporylated (P-CREB) and total (T-CREB) form of CREB and BDNF content. Each column represents the mean + S.E.M. of 4 experiments. Statistical analysis was performed by two-way ANOVA, followed by Duncan's test. *P<0.05 and **P<0.01 as compared with the control group (Sham-vehicle) and # P<0.05 as compared with the O.B.-vehicle group. The bands on the top show the CREB expression on phosphorylated form (upper bands) and total form (lower bands) and BDNF content (upper bands) as compared to β actin (lower bands) referent to sham/vehicle (S-Ct), sham/extract 10 mg/kg (S-E10), sham/extract 30 mg/kg (S-E30), OB/vehicle (B-Ct), OB/extract 10 mg/kg (B-E10) and OB/extract 30 mg/kg (B-E30) groups.

Figure 1.

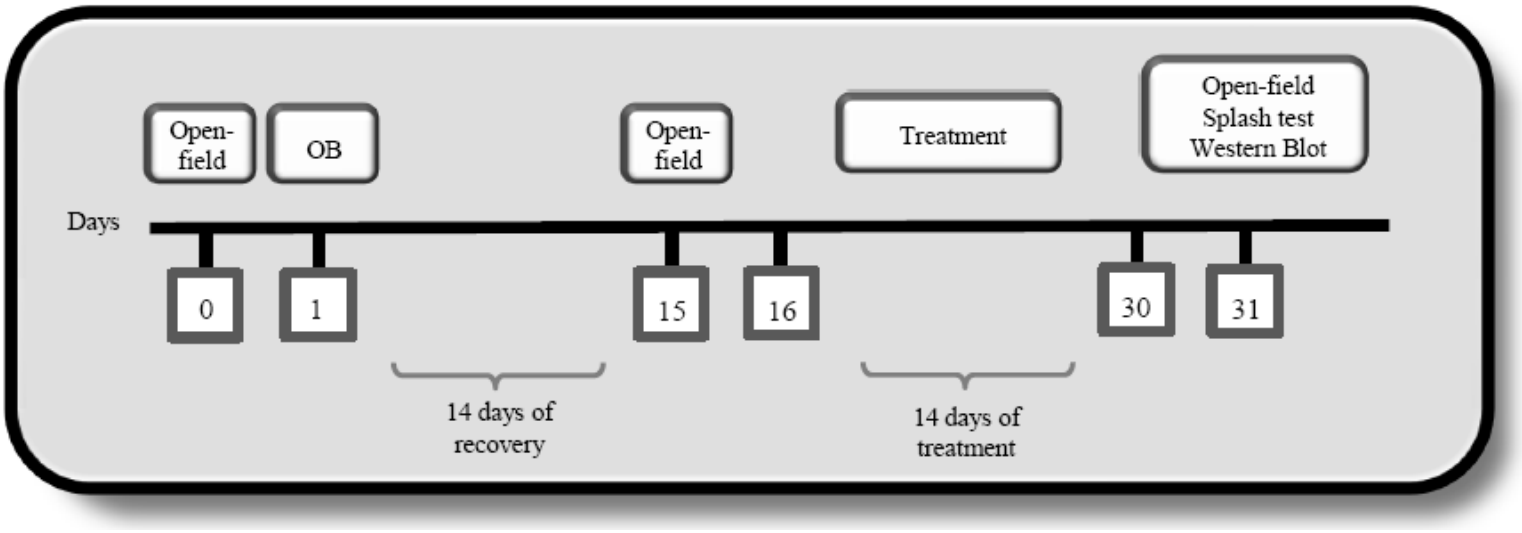

Open-field
OB
Open-field
Treatment
Open-field
Splash test
Western Blot
Days
0
1
15
16
30
31
14 days of recovery
14 days of treatment

Figure 2.

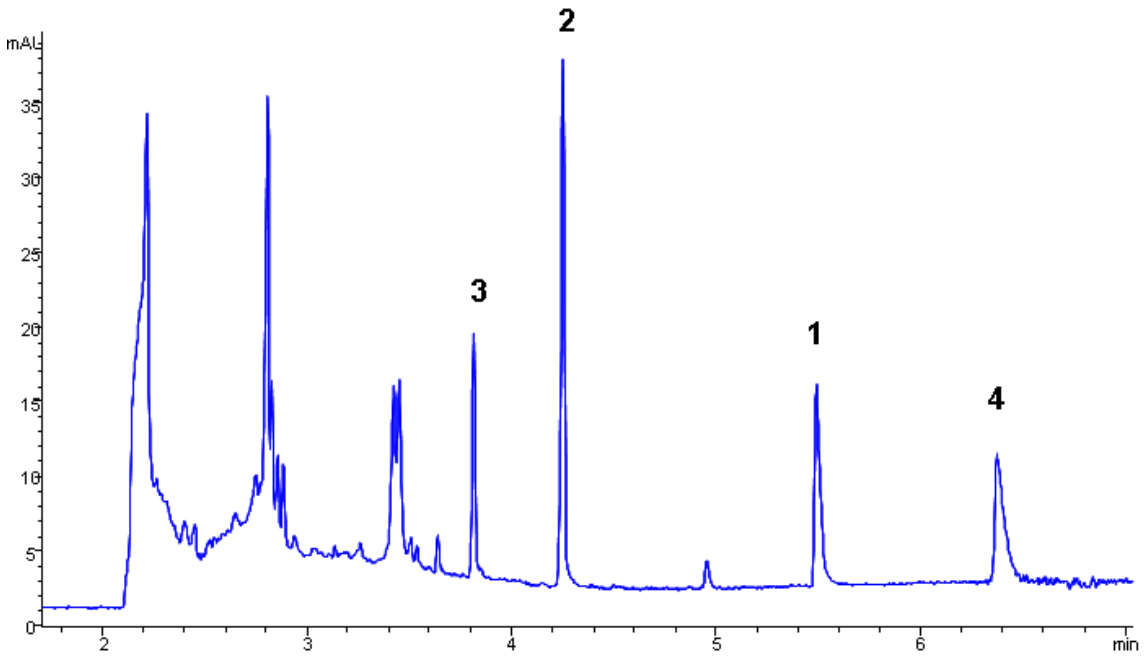


Figure 3.

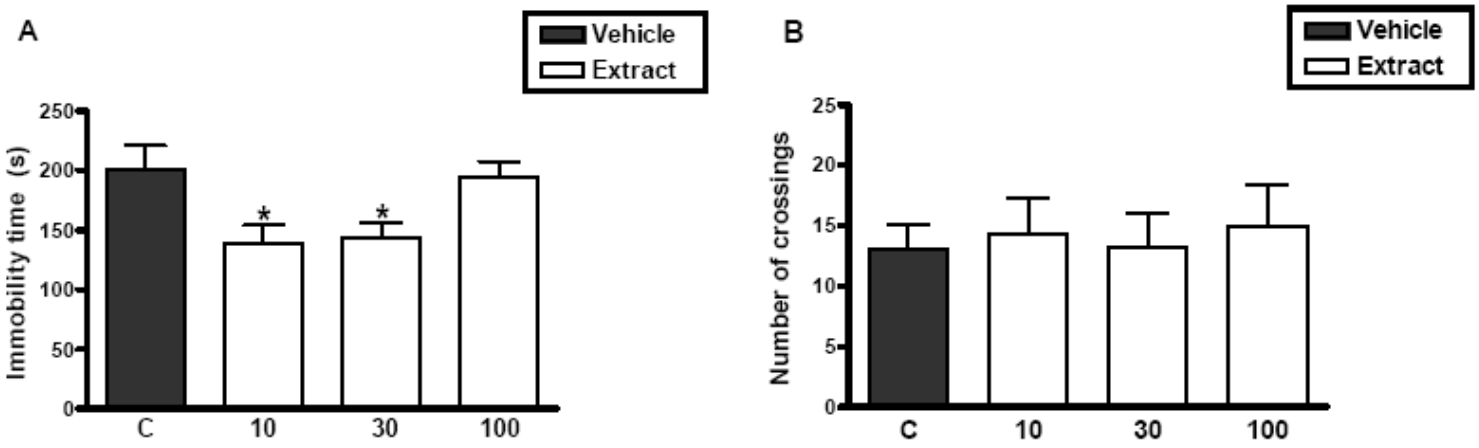

A
Vehicle
Extract
Immobility time (s)
250
200
150
100
50
0
*
*
C
10
30
100
B
Vehicle
Extract
Number of crossings
25
20
15
10
5
0
C
10
30
100

Figure 4.

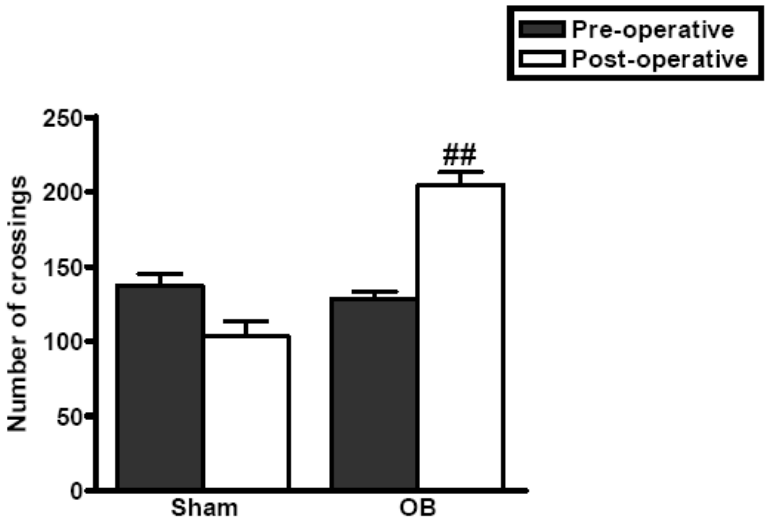

Pre-operative
Post-operative
Number of crossings
250
200
150
100
50
0
##
Sham
OB

Figure 5.

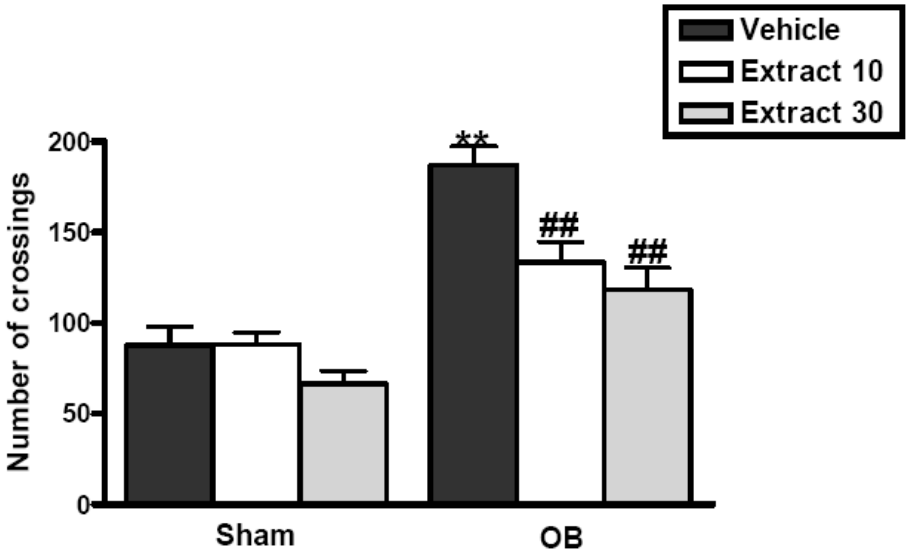

Vehicle
Extract 10
Extract 30
Number of crossings
200
150
100
50
0
**
##
##
Sham
OB

Figure 6.

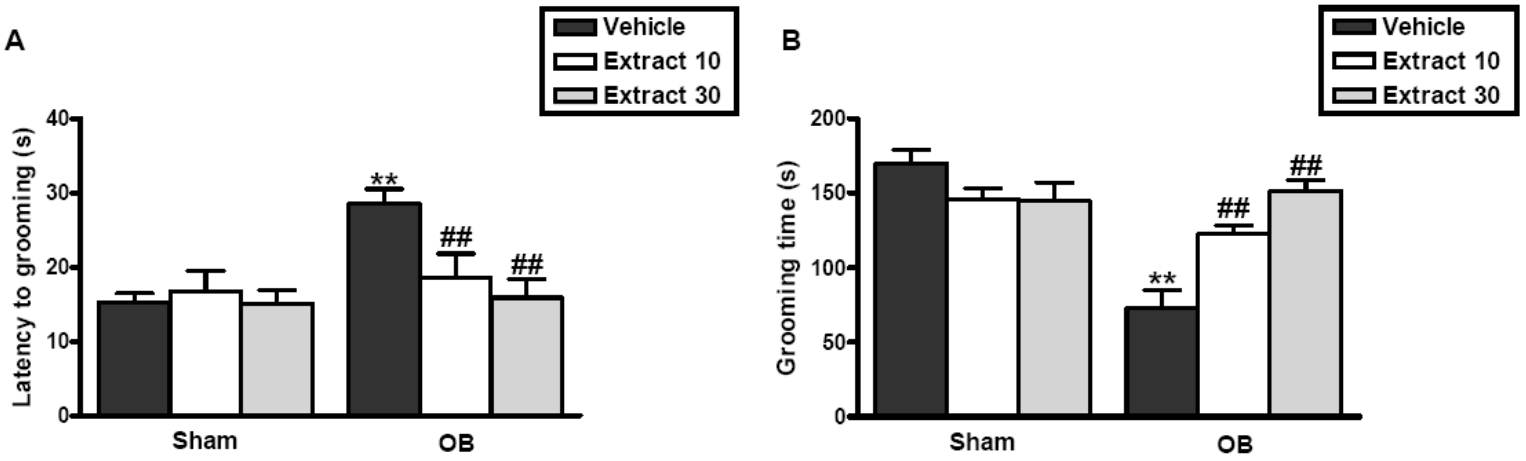

A
Vehicle
Extract 10
Extract 30
Latency to grooming (s)
40
30
20
10
0
**
##
##
Sham
OB
B
Vehicle
Extract 10
Extract 30
Grooming time (s)
200
150
100
50
0
**
##
##
Sham
OB

Figure 7.

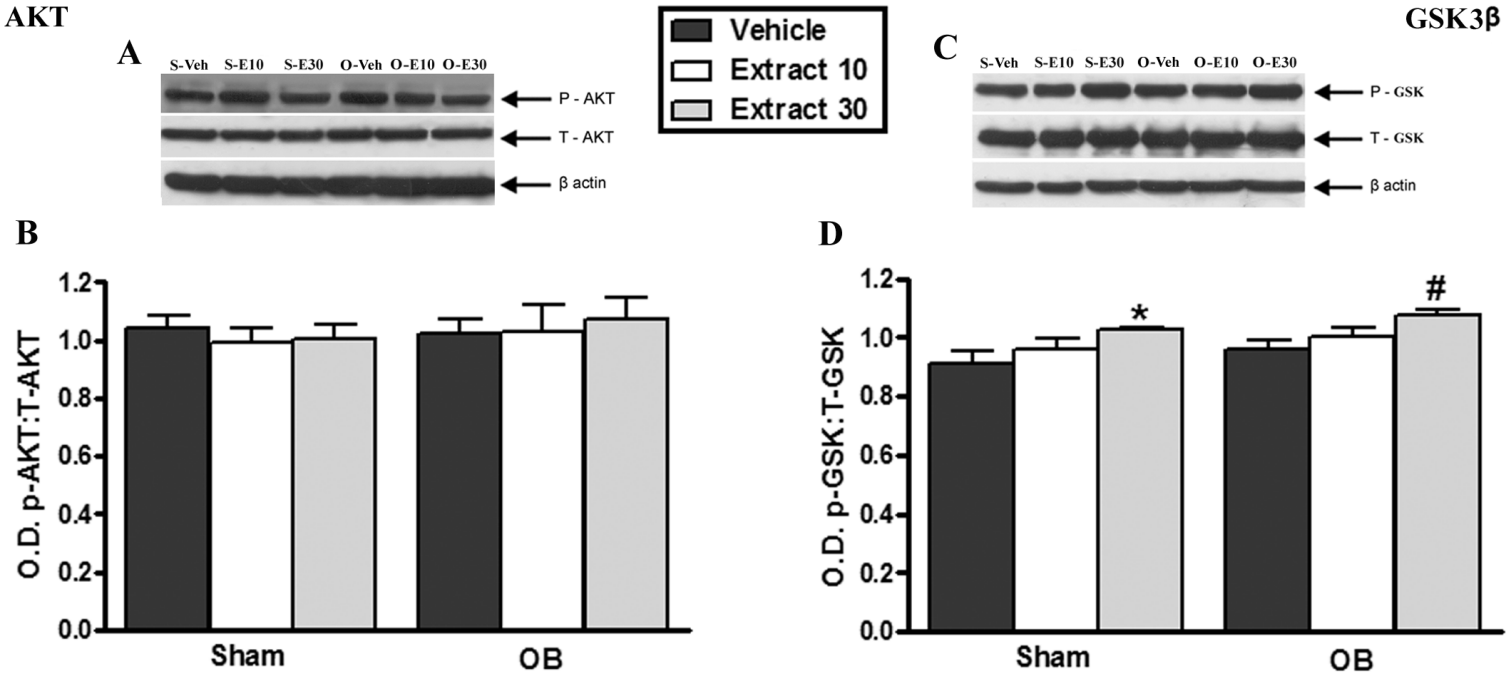

AKT
A
S-Veh S-E10 S-E30 O-Veh O-E10 O-E30
P - AKT
T - AKT
β actin
Vehicle
Extract 10
Extract 30
GSK3β
C
S-Veh S-E10 S-E30 O-Veh O-E10 O-E30
P - GSK
T - GSK
β actin
B
O.D. p-AKT:T-AKT
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
OB
D
O.D. p-GSK:T-GSK
*
#
Sham
OB

Figure 8.

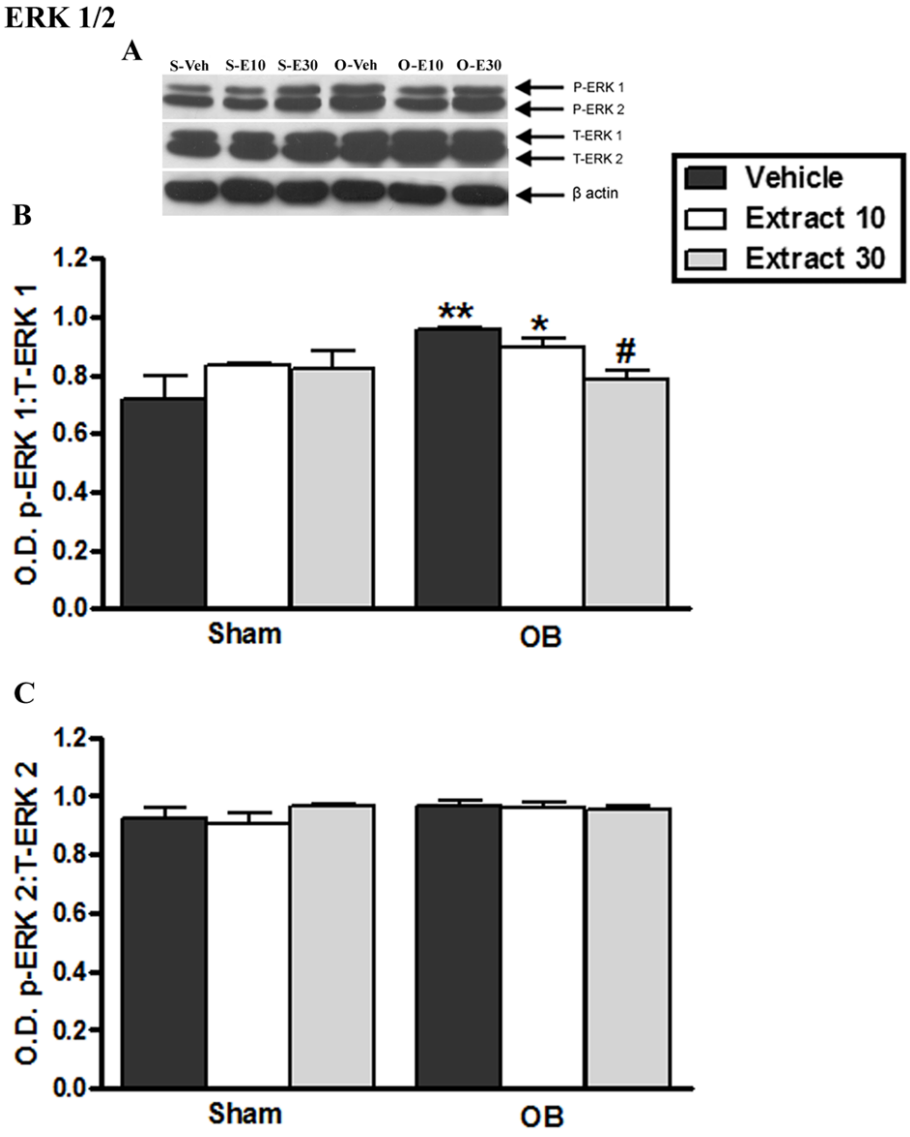
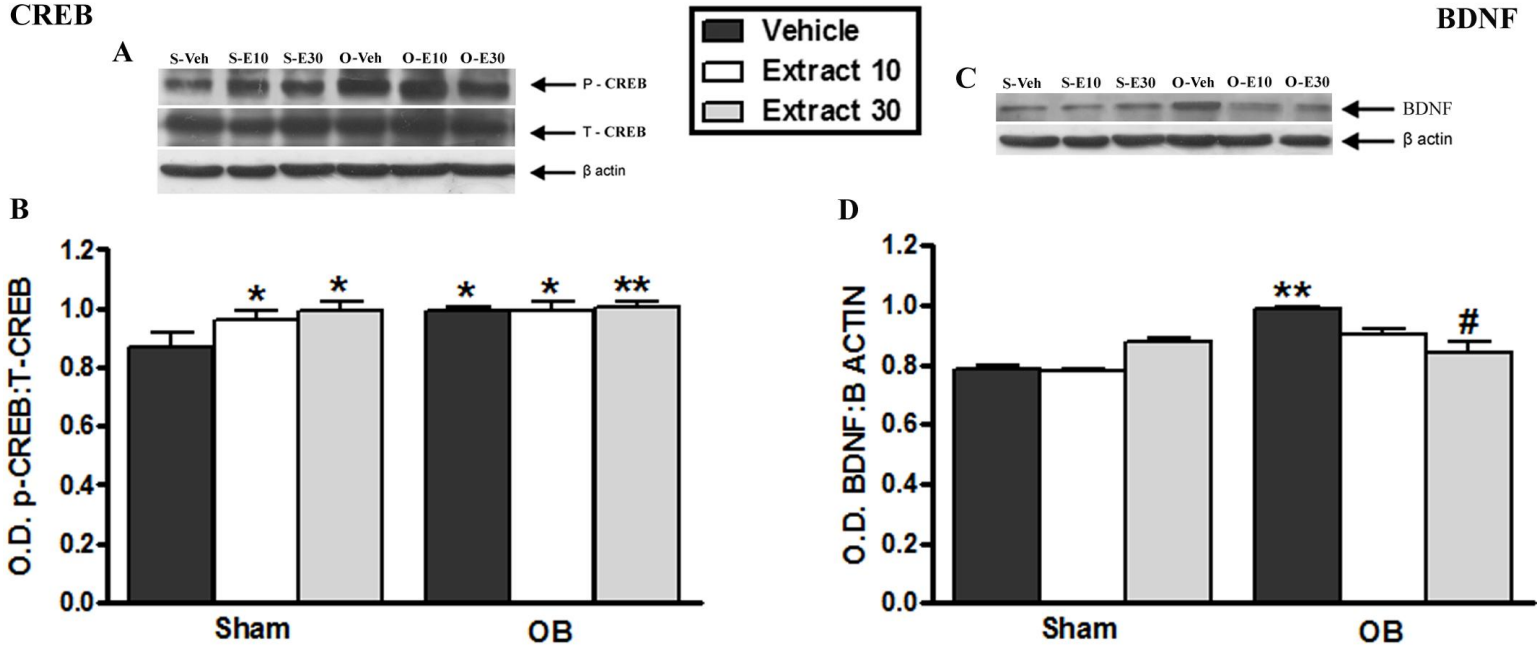

ERK 1/2
A
S-Veh
S-E10
S-E30
O-Veh
O-E10
O-E30
P-ERK 1
P-ERK 2
T-ERK 1
T-ERK 2
β actin
B
Vehicle
Extract 10
Extract 30
O.D. p-ERK 1:T-ERK 1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
#
Sham
OB
C
O.D. p-ERK 2:T-ERK 2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
OB

Figure 9.


CREB
BDNF
A
S-Veh S-E10 S-E30 O-Veh O-E10 O-E30
P - CREB
T - CREB
β actin
Vehicle
Extract 10
Extract 30
C
S-Veh S-E10 S-E30 O-Veh O-E10 O-E30
BDNF
β actin
B
O.D. p-CREB:T-CREB
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
*
**
Sham
OB
D
O.D. BDNF:B ACTIN
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
#
Sham
OB

5.2. Manuscrito 2

Fluoxetine modulates hippocampal cell signaling pathways implicated in neuroplasticity and abolishes depressive-related behavior induced by olfactory bulbectomy in mice

Andiara E Freitas, Daniele G Machado, Josiane Budni, Vivian B Neis, Grasiela O Balen, Mark W Lopes, Luiz F de Souza, Alcir L Dafre, Rodrigo B Leal, Ana Lúcia S. Rodrigues*

Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário – Trindade - 88040-900, Florianópolis-SC, Brazil

* Corresponding author. Tel.: +55 (48) 3721-5043; fax: +55 (48) 3721-9672.

E-mail address: analucia@mbox1.ufsc.br or ana.rodrigues@pq.cnpq.br

Abstract: The olfactory bulbectomy (OB) is a well-established animal model of depression capable of detecting antidepressant activity following chronic treatment and results in behavioral and biochemical changes reminiscent of various symptoms in depression. In this study we investigated the ability of the repeated (14 days) p.o. administration of the classical antidepressant fluoxetine (10 mg/kg) to reverse OB-induced alterations in behavior (hyperactivity and anhedonic behavior) and in hippocampal cell signaling pathways involved in synaptic plasticity. OB caused a significant increased of ERK1 and CREB (Ser^{133}) phosphorylation and BDNF immunocontent. Fluoxetine prevented the OB-induced hyperactivity, anhedonia (loss of motivational and self care behavior) and increased either ERK1 and CREB phosphorylation or BDNF immunocontent. Moreover, fluoxetine caused a significant decrease in ERK2 phosphorylation in OB-group. Akt and GSK-3β phosphorylation were not altered in any experimental condition. In conclusion, the present study shows that OB can induce significant behavioral alterations that are accompanied by activation of hippocampal signaling pathways, named ERK1/CREB/BDNF, which are involved in the synaptic plasticity. Conversely, fluoxetine prevented OB-induced behavioral changes and abrogated the activation of ERK1/CREB/BDNF in the hippocampus. Taken together, our results extend literature data about behavioral and neurochemical alterations induced by OB and suggest possible mechanism underlying the antidepressant effect of fluoxetine in this model.

Keywords: antidepressant; fluoxetine; olfactory bulbectomy; ERK1/2; CREB; BDNF.

1. Introduction

Depression is a debilitating disease with a high prevalence and social cost [1]. Among antidepressant drugs, selective serotonin reuptake inhibitors (SSRIs), including fluoxetine, are the most widely prescribed [2]. Despite their well-established efficacy, the molecular mechanisms underlying the therapeutic action of SSRIs remain unclear. Neurobiological theories have proposed that the efficacy of fluoxetine may be due to alterations in various signaling pathways regulating cellular plasticity and survival [3]. Fluoxetine treatment increases the rate of adult hippocampal proliferation after chronic but not acute administration [4]. This idea corroborates with the neurotrophin hypothesis of depression, which invokes increased expression of brain-derived-neurotrophic factor (BDNF), resulting from augmented neural activity elicited by antidepressant medications [5]. BDNF activates a variety of signaling cascades, including the phosphatidylinositol 3′-kinase (PI-3K)-Akt [6] and the mitogen-activated protein kinase (MAPK) pathway [7]. Several MAPK cascades have been characterized, of which the best studied MAPKs are the extracellular signal-regulated kinases ERK1 and ERK2. These pathways, as well as several others that promote neuronal survival, converge on a transcriptional regulator, cyclic-AMP responsive-element binding protein (CREB). A number of growth factors and hormones have been shown to stimulate the expression of cellular genes when CREB is activated by phosphorylation at Ser^{133} [8].

Several lines of evidence have shown that depressive disorders are related to the activation of the enzyme glycogen synthase kinase-3β (GSK-3β), through a failure on its mechanism of inhibition mediated by phosphorylation [9]. Protein kinases such as Akt/protein kinase B (PKB), protein kinase A (PKA) and protein kinase C (PKC) regulate negatively the activity of GSK-3b by phosphorylation at the N-terminal Ser^{9}

[9]. In general, increased activity of GSK-3b is proapoptotic, whereas inhibiting GSK-3β prevents apoptosis. GSK-3β has also recently been identified as a common target for SSRIs, tricyclic antidepressants (TCAs) and antipsychotics [10–12].

The antidepressant efficacy of different classes of compounds is currently assessed by the tail suspension test (TST) and the forced swimming test (FST) [13]. However, a major limitation of these behavioral tools is that antidepressant activity is detected following acute administration, whereas in clinical depression, several days or weeks elapse before a therapeutic effect is observed [14]. A widely used animal model of depression is the olfactory bulbectomy (OB), which is able to detect antidepressant activity almost exclusively following chronic, but not acute antidepressant treatment [15]. OB has been proposed as an animal model of depression in terms of construct validity, since it induces alterations in behavior, and in the endocrine, immune and neurotransmitter systems that reproduces many of those seen in depressive patients [16, 17].

It was previously reported that rats subjected to OB exhibit a decrease in the vulnerability of hippocampal pyramidal neurons to excitotoxic injury [18] suggesting a possibility that OB affects neurotrophic factor signaling in the hippocampus. However, the cellular and molecular mechanism underlying this hippocampal neuroplasticity in response to OB is poorly studied. Therefore, the present study aimed to verify the effect of OB alone on the modulation of signaling targets associated with hippocampal synaptic plasticity such as Akt, GSK-3β, ERK1/2, CREB and BDNF. Moreover, the ability of fluoxetine to reverse OB-induced alterations on behavior and on the hippocampal signaling parameters was also evaluated.

2. Methods

2.1. Animals

Female Swiss mice (2 month old, 30-40g) were maintained at constant room temperature (20-22°C) with free access to water and food, under a 12:12h light:dark cycle (lights on at 07:00 h). The cages were placed in the experimental room 24 h before the test for acclimatization. All manipulations were carried out between 9:00 and 17:00 h, with each animal used only once. The procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee. All efforts were made to minimize animal suffering and the number of animals used in the experiments.

2.2. Surgical procedure

After a 2-week acclimatization period, bilateral OB was performed by suction method described previously [19]. Animals were randomly divided into two groups: OB and sham-operated animals. Briefly, mice were anesthetized with xylazine (6 mg/kg, i.p.) in combination with ketamine (100 mg/kg, i.p.) diluted in saline. An incision was made in the skin overlying the skull, and, after exposure of the skull, holes were drilled on both sides of the mid-line. Then the olfactory bulbs were bilaterally aspired bilaterally aspirated by blunt hypodermic needle (with for 1.0 to 1.2 cm long and with a rounded tip of 0.80 to 1.2 mm of diameter) attached the syringe (10 ml) for the suction, taking care not to cause damage to the frontal cortex. The holes were filled with swab in order to stop the bleeding and covered with dental cement. All surgical procedure was carried out employing alcohol 70% to eliminate contaminations. Sham-operated animals were treated in the same way except the bulbs were left untouched. Mice were allowed to recover under warming help with body temperature maintenance. The animals were given 14 days to recover following surgery prior to treatments.

2.3. Drugs and treatment

Fluoxetine (10 mg/kg, Sigma Chemical Co.) was dissolved in distilled water and administered by oral route (p.o.) by gavage. A control group received distilled water as vehicle.

Two weeks after surgery, fluoxetine (10 mg/kg, p.o.) was administered once daily for 14 days. Animals were assigned to the following groups: (a) sham/vehicle, (b) sham/fluoxetine, as the control groups; (c) OB/vehicle, and (d) OB/fluoxetine. Number of mice per group was 9-12.

2.4. Open-field test

The OB model of depression reproduces in preclinical assays the psychomotor agitation consistent with that is observed in agitated depression [20]. To assess the effects of OB on locomotor activity, mice were evaluated in the open-field paradigm as previously described [21]. The test was consecutively performed in 3 time periods: pre-surgically, 2 weeks after surgery, and post-treatment (after 2 weeks of fluoxetine or water p.o. treatment). The number of squares crossed with all paws (crossings) was counted in a 6 min session. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.5. Splash test

The splash test was carried out 24 h after the last repeated drug administration as described by Isingrini and co-workers [22], with minor modifications. The test consists of squirting a 10% sucrose solution on the dorsal coat of a mouse placed individually in clear Plexiglas boxes (9 x 7 x 11 cm). Because of its viscosity, the sucrose solution dirties the mouse fur and animals initiate grooming behavior. After applying sucrose solution, the latency to grooming and the grooming time were manually recorded for a period of 5 minutes as an index of self-care and motivational behavior [23,24]. The

apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

2.6. Western Blot

After two weeks of treatment, and 24 h after the last administration of fluoxetine or vehicle by oral rote, mice were decapitated. Brains were removed and the lesion was estimated macroscopically immediately after brain removal; all brains with incomplete surgery or cortex damage were discarded from the experiment. The hippocampus was rapidly dissected and placed in cold saline solution. Western blot analysis was performed as previously described [25,26]. Briefly, hippocampal tissue were mechanically homogenized in 400 µl of Tris-base 50 mM pH 7.0, EDTA 1 mM, sodium fluoride 100 mM, PMSF 0.1 mM, sodium vanadate 2 mM, Triton X-100 1%, glycerol 10%, and then incubated for 30 min in ice. Lysates were centrifuged (10000 x g for 10 min, at 4ºC) to eliminate cellular debris, and supernatants diluted 1/1 (v/v) in Tris-base 100 mM pH 6.8, EDTA 4 mM, SDS 8%, glycerol 16%. Protein content was estimated by the method previously described [27] and concentration calculated by a standard curve with bovine serum albumin. To compare signals obtained, the same amount of protein (70 µg per lane) for each sample was electrophoresed in 10% SDS-PAGE minigels (after addition of bromophenol blue 0.2% and β-mercaptoethanol 8%) and transferred to nitro-cellulose or polyvinylidene fluoride (PVDF) membranes. To verify transfer efficiency process, gels were stained with Coomassie blue (Coomassie blue R-250 0.1%, methanol 50%, acetic acid 7%) and membranes with Ponceau 0.5% in acetic acid 1%.

After this process, blots were incubated in a blocking solution (5% non-fat dry milk in Tris buffer saline solution, TBS) for 1 h at room temperature and then probed at 4ºC with anti-phospho-Akt (Sigma Chemical Co., 1:1000), anti-phospho-CREB (Ser^{133})

(Cell Signaling, 1:1000), anti-phospho-GSK-3β (Cell Signaling, 1:1000), anti-phospho-ERK1/2 (Cell Signaling, 1:2000), anti-ERK1/2 (Sigma Chemical, 1:40000), anti-Akt (Sigma Chemical, 1:1000), anti-GSK-3β (Cell Signaling, 1:1000), anti-CREB (Cell Signaling, 1:1000) and anti-BDNF (Millipore, 1:1000), all in TBS containing 0.05% Tween 20 (TBS-T). Next, the membranes were incubated with anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibody (1:5,000) for 1 h and the immunoreactive bands were developed by chemiluminescence (LumiGLO®, Cell Signaling, Beverly, MA, USA). All blocking and incubation steps were followed by three washes (5 min) of the membranes with TBS-T. In order to detect phosphorylated and total forms of ERK1/2, Akt and CREB in the same membrane, the immunocomplexes were stripped as previously described [28]. Briefly, membranes were washed once with double deonized water (5 min), followed by incubation with NaOH 0.2 M (5 min), washing with double deonized water (5 min) and with TBST (10 min). The membranes stripped of immune complexes were blocked and followed the same steps described above. In order to ascertain the same protein load for each experimental group the house keeping protein, β-actin, was evaluated using a mouse anti-β-actin antibody (Santa Cruz, 1:1000) and anti-mouse HRP-conjugated (Millipore 1:4000) as a secondary antibody.

The optical density (O.D.) of the bands was quantified using Scion Image software®. The phosphorylation level of Akt, CREB, GSK-3β and ERK1/2 were determined as a ratio of O.D. of phosphorylated band/O.D. of total band. The immunocontent of BDNF were determined making the relationship between the O.D of BDNF band/O.D of β actin band.

A diagram of all experimental schedule is given in Fig. 1.

2.7. Statistical analysis

Comparisons between experimental and control groups were performed by one-way or two-way ANOVA followed by Duncan's HSD test when appropriate. Pearson's correlation analysis was performed to investigate any possible interrelation among behavioral and neurochemical parameters. $P<0.05$ was considered significant.

3. Results

3.1. Effect of a two-week treatment with fluoxetine on hyperactivity and anhedonic behavior induced by OB

In order to validate the OB model, mice were submitted to the open-field test in the pre- and post-operative periods. The sham-operated mice presented no significant alteration in the post-operative period as compared to the pre-operative period (data not shown). As predicted by the OB model, bulbectomized mice presented a higher number of crossings in the open-field test in the post-operative period compared to the pre-operative period (data not shown). Fig. 2A shows that the repeated administration of fluoxetine (10 mg/kg, p.o.) was able to reverse the hyperactivity induced by OB in the open-field apparatus, but without altering the behavior of sham operated animals. A two-way ANOVA revealed significant differences of OB [$F(1,34)=22.15$, $P<0.01$], treatment [$F(1,34)=18.26$, $P<0.01$] and OB x treatment interaction [$F(1,34)=5.62$, $P<0.05$]. Post hoc analyses indicated that fluoxetine treatment for 14 days to bulbectomized mice prevented the hyperactivity caused by OB.

The results depicted in Fig. 2B illustrates that the increase in latency to grooming elicited by OB was significantly blocked by fluoxetine (10 mg/kg, p.o.) treatment during 14 days, however no behavioral changes were observed in the sham operated animals. The two-way ANOVA revealed significant differences of OB [$F(1,35)=22.85$, $P<0.01$], treatment [$F(1,35)=15.96$, $P<0.01$] and OB x treatment

interaction [F(1,35)=4.67, P<0.05]. Post hoc analyses indicated that fluoxetine (10 mg/kg, p.o.) administered for 14 days to bulbectomized mice prevented the anhedonic behavior in the splash test produced by the surgery procedure. Also, Fig. 2C shows that the reduced grooming time caused by OB was significantly reversed by fluoxetine repeated treatment, once more without significant effects in the sham operated animals. A two-way ANOVA revealed significant differences of OB [F(1,35)=13.28, P<0.01], treatment [F(1,35)=8.90, P<0.01] and OB x treatment interaction [F(1,35)=9.77, P<0.01]. Post hoc analyses indicated that fluoxetine (10 mg/kg, p.o.) treatment (for 14 days) was able to prevent the anhedonic behavior (expressed by the increase in latency to grooming and the decrease in the grooming time) in the splash test caused by OB.

3.2. Akt, GSK-3β, ERK1/2 and CREB phosphorylation and BDNF immunocontent

Western blot analysis from hippocampal tissue homogenates showed that neither the OB procedure nor the repeated treatment with fluoxetine altered Akt or GSK-3β phosphorylation (Fig. 3B and D, respectively). The two-way ANOVA revealed no differences for OB [F(1,12)=0.56, P=0.47], treatment [F(1,12)=0.006, P=0.94] and OB x treatment interaction [F(1,12)=1.50, P=0.24] for Akt phosphorylation and for OB [F(1,12)=0.16, P=0.69], treatment [F(1,12)=0.72, P=0.41] and OB x treatment interaction [F(1,12)=0.52, P=0.48] for GSK-3β phosphorylation.

The OB, but not vehicle or fluoxetine sham operated animals, produced a slight increase in ERK1 phosphorylation (Fig. 4B). This effect was not apparent for ERK2 phosphorylation (Fig. 4C). Fluoxetine (10 mg/kg, p.o.) treatment for 14 days not only prevented, but significantly decreased ERK1 and ERK2 phosphorylation (Fig. 4B and C). Two-way ANOVA revealed a significant effect of OB [F(1,12)=10.81, P<0.01], treatment [F(1,12)=21.84, P<0.01] and OB x treatment interaction [F(1,12)=46.41, P<0.01] for pERK1 and also a significant main effect of treatment [F(1,12)=15.84,

P<0.01] and OB x treatment interaction [F(1,12)=11.29, P<0.01], but not of OB [F(1,12)=3.79, P=0.07] for pERK2 phosphorylation.

Alike ERK1 phosphorylation, a similar pattern of changes was observed by repeated treatment with fluoxetine on CREB phosphorylation and BDNF immunocontent in OB-mice (Fig. 5). It is remarkable that fluoxetine treatment did not induce these changes in sham operated animals. OB caused an increased p-CREB/total CREB ratio. This increased phosphorylation was significantly blocked by fluoxetine (10 mg/kg, p.o.) treatment for 14 days (Figure 5B). Two-way ANOVA revealed a significant effect of OB [F(1,12)=4.50, P<0.05], treatment [F(1,12)=7.43, P<0.01], and OB x treatment interaction [F(1,12)=7.47, P<0.01]. Fig. 5D shows that bulbectomized mice presented an increased BDNF immunocontent, which was prevented by the repeated treatment with fluoxetine that decreased BDNF expression. The two-way ANOVA revealed significant effects of treatment [F(1,12)=13.30, P<0.01] and OB x treatment interaction [F(1,12)=30.75, P<0.01], but no significant main effect of OB [F(1,12)=0.35, P=0.56].

Worth of note is the negative Pearson's correlation (Table 1) between either number of crossings and grooming time (P<0.001) or grooming and latency to grooming (P<0.05). From the signaling molecules studied, only CREB phosphorylation correlated with the number of crossings. It is interesting to note that a significant correlation was found between pERK1 and BDNF (P<0.01) as well as pCREB and BDNF (P<0.05). GSK-3β and Akt did not correlate with any variable studied (data not shown).

4. Discussion

The preset work further contributes to the understanding of the mechanisms underlying the antidepressant-like action of fluoxetine in a well-established animal

model of depression, the olfactory bulbectomy. In addition, it extends literature data regarding OB influence on the expression of proteins implicated in neuroplasticity and cellular survival. As opposed to the majority of literature studies that investigate behavioral and neurochemical effects of OB in male rats or mice, in the present study female mice were chosen considering that more women suffer depression than men [29]. The OB model of depression standardized in our laboratory reproduced several behavioral changes reported in literature, mainly the increased locomotor activity in the open-field paradigm and the anhedonic behavior in the splash test, providing validation for the model. The treatment for 14 days with fluoxetine in bulbectomized mice was effective in preventing the hyperactivity and the anhedonic behavior induced by OB. Fluoxetine caused a significant decrease in hippocampal ERK2 phosphorylation in OB-group. In addition, it was able to reverse the increased ERK1 and CREB phosphorylation and BDNF immunocontent in the hippocampus. Neither fluoxetine nor OB caused alterations in Akt (Ser^{473}) and GSK-3β (Ser^{9}) phosphorylation. Altogether, the results extend literature data about behavioral and biochemical alterations induced by OB and suggests that the antidepressant action of fluoxetine in this model appears to be related to the modulation of hippocampal ERK1/2, CREB and BDNF signaling pathways. However, changes in GSK-3β and Akt phosphorylation were not observed in OB and/or fluoxetine treatment.

Fluoxetine is an antidepressant drug that is generally assumed to exert its therapeutic effect by acting as a selective serotonin (5-HT) reuptake inhibitor (SSRI) causing an increase in the concentration of 5-HT in the synaptic cleft and thereby enhancing synaptic 5-HT signaling [2]. Olfactory bulbectomy is an established and useful model for studying depression and mechanisms of action of antidepressants [16,17]. This model supplants the major limitations of inescapable stress models such as

the TST and FST since is capable of detecting antidepressant activity almost exclusively following chronic drug treatment, and results in behavioral changes reminiscent of various symptoms in depression including learning and memory dysfunctions, psychomotor agitation, altered avoidance behavior and anhedonia [16].

Augmentation of the locomotor activity in the open-field paradigm is a parameter that provides validation for the procedure surgery [16] and reflects the psychomotor agitation, a key-symptom to diagnosis of agitated depression [30]. In the present work, the olfactory bulbs destruction produced a significant hyperlocomotion consistent with clinical symptoms of agitated depression that was prevented by the treatment (14 days) with fluoxetine at dose of 10 mg/kg (p.o.). The ability of fluoxetine to block symptoms of agitated depression corroborates with several literature data and with a previous study from our group [31,32] and is similar to the result reported for other antidepressant drugs such as amitriptyline, citalopram [33] and venlafaxine [34].

Anhedonia is an affective symptom related to the inability to experience pleasure which was assessed in the present study by the splash test. In this test, an increase in the latency to grooming as well as a decreased in the total grooming time indicates a depressive behavior [35]. Furthermore, a reduction in the grooming time in the splash test is considered to parallel the motivational and apathetic behavior observed in depression [23]. Our results are in line with these findings by showing that the destruction of the olfactory bulbs induced anhedonia and a loss of motivational and self care behavior evidenced by the increased latency to grooming and the decreased grooming time. Noteworthy, these behavioral alterations were not observed in bulbectomiuzed mice treated with fluoxetine (10 mg/kg, p.o.), indicating that it is effective in preventing anhedonic symptoms induced by OB, a result that confirm previous data from our group. In addition, our results are in agreement with studies

which show that fluoxetine administration is effective in preventing anhedonia induced by chronic unpredictable stress [36], social stress [37] dexamethasone-induced disruption of the hypothalamic-pituitary-adrenal axis [38] and interferon-α [39].

Grooming was also negatively correlated to the number of crossings in the open-field, in accordance to data presented by Lalonde and Strazielle [40] using a hyperactive mouse model, reinforcing the association of these behavioral end points in the OB model of depression. The behavioral responses latency to grooming and grooming were negatively correlated (Table 1), validating the ANOVA findings, i.e., OB increased latency to grooming and decreased grooming time.

There is a growing amount of evidence showing that signaling pathways related to synaptic plasticity may be involved in the antidepressants mechanism of action and the pathophysiology of depression [41,42]. Despite fluoxetine efficacy in OB is well-established, the molecular mechanisms underlying its therapeutic action remain to be established. The present study addresses this issue by investigating the involvement of hippocampal Akt, GSK-3β, ERK-1/2, CREB and BDNF-mediated signaling pathways in the OB model of depression.

GSK-3 is a serine/threonine protein kinase that is highly expressed in brain [43], and plays important roles in synaptic plasticity [44], neurogenesis [45] and antidepressant-like behavior [46]. It is generally considered constitutively active and found in two isoforms, α and β. GSK-3β has been suggested to be implicated in the pathogenesis of bipolar disorder, schizophrenia and depression. This enzyme is proposed to be an important target for drugs used in the therapy of these disorders [9]. GSK-3β normally undergoes inhibitory regulation by upstream protein kinases, such as Akt/PKB, protein kinase A (PKA), and protein kinase C (PKC) [47], via phosphorylation at N-terminal Ser^9 [44]. Akt/PKB is a serine/threonine kinase that

phosphorylates and regulates the function of many cellular proteins involved in processes related to metabolism, apoptosis, proliferation and neuroplasticity [48], including GSK-3β. Akt/PKB contains two regulatory phosphorylation sites, Thr^{308} in the activation loop within the kinase domain and Ser^{473} in the C-terminal regulatory domain [49]. The implication of Akt and GSK-3β in the behavioral effects elicited by OB is not well established. In the present study, however, neither fluoxetine nor OB produced alterations on Akt (Ser^{473}) or GSK-3β (Ser^{9}) phosphorylation in the hippocampus, indicating that the depressive-like behavior of bulbectomized mice seems not to be associated with hippocampal Akt-GSK-3β-mediated signaling pathway. Similarly, these results suggest that these molecular targets, at least in the hippocampus, are not underlying the antidepressant effect of fluoxetine in bulbectomized mice. Since we are working with whole hippocampus our results are not necessarily contradictory to a study by Shioda et al. [50] that showed decreased phospho-Akt immunoreactivity and Akt levels in the dentate gyrus of the hippocampus following OB in mice.

ERK1 and ERK2 are member of the mitogen-activated protein kinase (MAPK) family that have been involved in the modulation of cell survival/death, cellular growth and cell differentiation [51–53]. Moreover, ERK1/2 may modulate hippocampal plastic events such as long term potetiation (LTP), long term depression (LTD) and memory [54,55]. ERK1/2 are activated by BDNF binding at the tropomyosin-related kinase B receptor (TrkB) via the Ras-Raf-MAPK kinase (MEK1/2)-ERK1/2 cascade, inducing nuclear translocation and phosphorylation of target transcription factors. When activated, the phosphorylation state of ERK1/2 primarily regulates neuronal growth, differentiation and apoptosis [53]. A growing body of evidence indicates that ERK pathway participates in the neuronal modulation of depression [56,57]. Furthermore, it has been reported that stress-induced depressive-like behaviors are correlated with an

increase in ERK1/2 phosphorylation at hippocampus. Chronic treatment with desipramine prevented this depressive-like behavior and ERK1 phosphorylation [58], suggesting that the blockade of this signaling pathway might represent an antidepressant mechanism. Noteworthy, a study by Galeotti and Ghelardini [59] showed ERK1/2 pathway as an important factor in vulnerability to depressive illnesses in stress. In their study, an activation of ERK1/2 in hippocampus and frontal cortex of animals exposed to behavioral despair paradigms was shown, as well as an antidepressant-like phenotype produced by the blockade of MAPK signaling. Therefore, it is possible that disruption in the regulation of ERK pathway could alter behavioral parameters and could participate in the molecular mechanisms in response to OB. In line with this, our results show that the increase in ERK1 phosphorylation produced by OB was significantly prevented by fluoxetine (10 mg/kg, p.o.) treatment, a finding that supports the notion that ERK1/2 inhibition produces antidepressant effects [41,59]. Reinforcing this hypothesis, treatment of bulbectomized mice with fluoxetine produced a significant decrease in hippocampal ERK2 phosphorylation. Thus, our results suggest that inhibition of ERK1/2 pathway appears to be a mechanism underlying the antidepressant effect of fluoxetine in OB mice.

Here we found that phosphorylation of the Ser133 site on cAMP response element-binding protein (CREB), a fundamental step for CREB activation, was stimulated in the hippocampus by OB. CREB is a nuclear transcription factor that in the brain is involved in neuroplasticity. Therefore, many physiological phenomena including memory, learning, neuroprotection, synaptic transmission, neuron survival, cell differentiation, axon growth can be modulated by CREB [60]. Ser^{133} on CREB is a well-known target for many protein kinases, including cyclic AMP-dependent protein kinase (PKA), protein kinase G (PKG), protein kinase C (PKC), Akt/PKB, MAPKs (via

Rsk) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK) II and IV [61]. Since these kinases are involved in different transduction system pathways, CREB plays an important role in integrating many signals into neuronal cells. It was recently reported that chronic psychosocial stress, an animal model of depression, stimulates CREB transcriptional activity in the brain that is prevented by treatment with the antidepressants such as imipramine [62], citalopram and fluoxetine [63]. Interestingly, in our study an increased CREB phosphorylation was observed in the hippocampus of OB animals, which may indicate an adaptive response to the disrupted neuronal connections. Similarly to our result, a recent study reported increased striatal CREB activity in OB rats [64]. The OB-induced increase in CREB phosphorylation was blocked by treatment (14 days) with fluoxetine at dose of 10 mg/kg (p.o.), corroborating with the notion that CREB could be a critical target implicated in the depressive-like effect induced by OB. It was possible to observe a good correlation ($P<0.01$) between pCREB and the number of crossings (Table 1), another indicative that CREB phosphorylation may be causally related to depression and to fluoxetine effects in the OB model. Indeed, there is compelling evidence showing that CREB is activated not only in response to the pro-growth and pro-survival stimuli but further in response to stressful stimuli [65]. In neurons, CREB is phosphorylated under conditions of hypoxia and oxidative stress, suggesting the activation of a cell survival program in response to harmful conditions [66].

The neurotrophic hypothesis of depression postulates that a neurotrophin deficit, especially hippocampal BDNF, is implicated in the pathogenesis of depression [67]. The elevated BDNF levels shown in our study following OB are in agreement with a study by [68] which showed that olfactory bulbs ablation leads to a significant increase in BDNF levels at hippocampus and frontal cortex as compared with sham-operated

mice. Additionally, the authors have shown that OB induces serotonergic dysfunctions, verified by a reduction of the molar ratio of 5-hydroxyindoleacetic acid to serotonin (5-HT) that indicates a decrease in 5-HT turnover [68]. Despite the original hypothesis indicating BDNF deficit in depression, the significant up-regulation of BDNF hippocampal immunocontent caused by OB may be an adaptive response that may change in more prolonged treatment. In line with this notion, rats subjected to OB exhibit a decrease in the vulnerability of hippocampal pyramidal neurons to excitotoxic injury [18], suggesting that OB improves neurotrophic factor signaling in the hippocampus. In addition, it is interesting to note that our results are in agreement with the increased hippocampal CREB phosphorylation. In fact, BDNF expression correlated to pERK1 ($P<0.01$) and pCREB ($P<0.05$), suggesting that they are common mediators of fluoxetine effects, which remains to be confirmed. Taking into account that fluoxetine is a SSRI antidepressant drug causing an increase in the concentration of 5-HT in the synaptic cleft, these findings partly explain the fluoxetine ability to reverse the augmentation of BDNF content and to block the behavioral alterations induced by OB.

5. Conclusion

The present study significantly supports literature data about behavioral modifications induced by OB and extends literature data by indicating some possible mechanisms underlying the antidepressant effect of fluoxetine in this pre-clinical animal model of depression. The OB procedure, standardized in our laboratory in female mice, caused hyperactivity in the open-field paradigm and anhedonic behavior in the splash test, providing validation for the model. OB induced a significant increase in ERK1/CREB/BDNF pathway in the hippocampus. In addition, the repeated treatment

with the antidepressant fluoxetine was effective in preventing hyperactivity and anhedonic behavior induced by OB. These effects of fluoxetine were associated with a reduction of ERK1/2 and CREB phosphorylation and BDNF immunocontent, but not with GSK-3β or Akt phosphorylation. The role of each of these neuroplastic and neuroprotective pathways deserves further studies to determine their direct relation to the antidepressant action of fluoxetine in the OB model.

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Legends to the figures:

Figure 1. Diagram of all experimental schedule. The effect of OB on locomotor activity was accessed in the open-field paradigm in 3 time periods: pre-surgically, after 2 weeks of surgery, and 2 weeks post-treatment. The animals were given 14 days to recover following surgery prior to treatments. Fluoxetine at dose of 10 mg/kg (p.o.) was administered once daily for 14 days. Twenty-four h after the last administration of fluoxetine, mice were submitted to open-field, splash test (tempo?) and hippocampus was rapidly dissected and prepared to western blot assay.

Figure 2. Effect of the repeated (14 days) treatment of bulbectomized mice with fluoxetine (10 mg/kg, p.o.) on the locomotor activity in the open-field test (panel A), latency to grooming (panel B) and grooming time (panel C) in the splash test. Each column represents the mean + S.E.M. of 9-12 animals. Statistical analysis was performed by two-way ANOVA, followed by the Duncan's HSD. **P<0.01 as compared with the control group (Sham-vehicle) and ## P<0.01 as compared with the OB-vehicle group.

Figure 3. Effect of repeated (14 days) treatment of bulbectomized mice with fluoxetine (10 mg/kg, p.o.) on Akt (panels A and B) and GSK-3β (panels C and D) phosphorylation. Panels A and C show a representative western blot. Quantitative analyses are illustrated in panels B and D. The data are expressed as ratio between phosporylated (P-Akt, P-GSK-3β) and total (T-Akt, T-GSK-3β) form of Akt and GSK-3β. Each column represents the mean + S.E.M. of 4 experiments. Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. The bands on the top show the Akt and GSK-3β expression on phosphorylated form (upper bands) and total form (lower bands) referent to sham/vehicle (S-Veh), sham/fluoxetine 10 mg/kg (S-F10), OB/vehicle (O-Veh), OB/fluoxetine 10 mg/kg (O-F10) groups.

Figure 4. Effect of repeated (14 days) treatment of bulbectomized mice with fluoxetine (10 mg/kg, p.o.) on ERK1 (panels A and B) and ERK2 (panels A and C) phosphorylation. Panel A shows a representative western blot. Quantitative analyses are illustrated in panels B and C. The data are expressed as ratio between phosporylated (P-ERK1 and P-ERK2) and total (T-ERK1 and T-ERK2) forms. Each column represents the mean + S.E.M. of 4 experiments. Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. *$P<0.05$ as compared with the control group (Sham-vehicle) and ## $P<0.01$ as compared with the OB-vehicle group. The bands on the top show the ERK1 and ERK2 expression on phosphorylated form (upper bands) and total form (lower bands) referent to sham/vehicle (S-Veh), sham/fluoxetine 10 mg/kg (S-F10), OB/vehicle (O-Veh), OB/fluoxetine 10 mg/kg (O-F10) groups.

Figure 5. Effect of repeated (14 days) treatment of bulbectomized mice with fluoxetine (10 mg/kg, p.o.) on CREB (panels A and B) phosphorylation and BDNF (panels C and

D) immunocontent. Panels A and C show a representative western blot. Quantitative analyses are illustrated in panels B and D. The data are expressed as ratio between phosporylated (P-CREB) and total (T-CREB) form of CREB and BDNF content. Each column represents the mean + S.E.M. of 4 experiments. Statistical analysis was performed by two-way ANOVA, followed by the Duncan's test. **P<0.01 as compared with the control group (Sham-vehicle) and ## P<0.01 as compared with the OB-vehicle group. The bands on the top show the CREB expression on phosphorylated form (upper bands) and total form (lower bands) and BDNF content (upper bands) as compared to β actin (lower bands) referent to sham/vehicle (S-Veh), sham/fluoxetine 10 mg/kg (S-F10), OB/vehicle (O-Veh), OB/fluoxetine 10 mg/kg (O-F10) groups.

Table 1.

Table 1. Pearson's correlation among selected variables.

Measures	Crossings post treatment	Latence to grooming	Grooming time	pERK1	pCREB
Latence to grooming	-0.01				
Grooming time	**-0.55*****	**-0.33***			
pERK1	0.06	0.02	-0.08		
pCREB	**0.68****	-0.18	-0.30	0.43	
BDNF	0.29	-0.18	-0.19	**0.70****	**0.56***

Significant at *P<0.05, **P<0.01 or***P<0.001.

Figure 1.

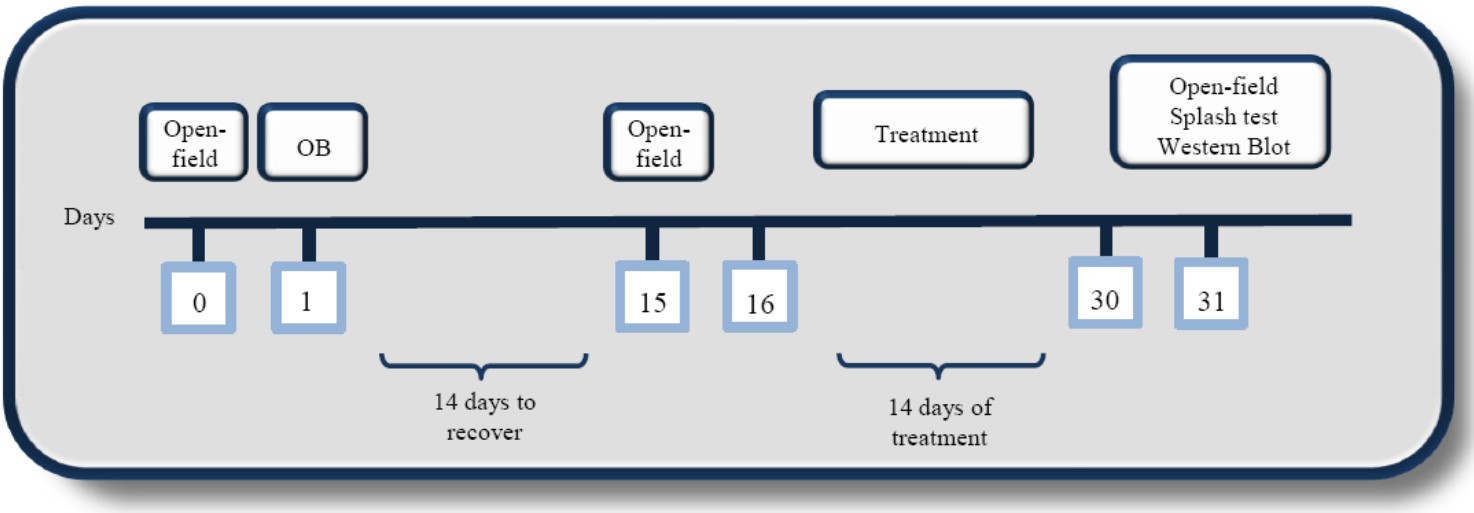

Open-field
OB
Open-field
Treatment
Open-field
Splash test
Western Blot
Days
0
1
15
16
30
31
14 days to recover
14 days of treatment

Figure 2.

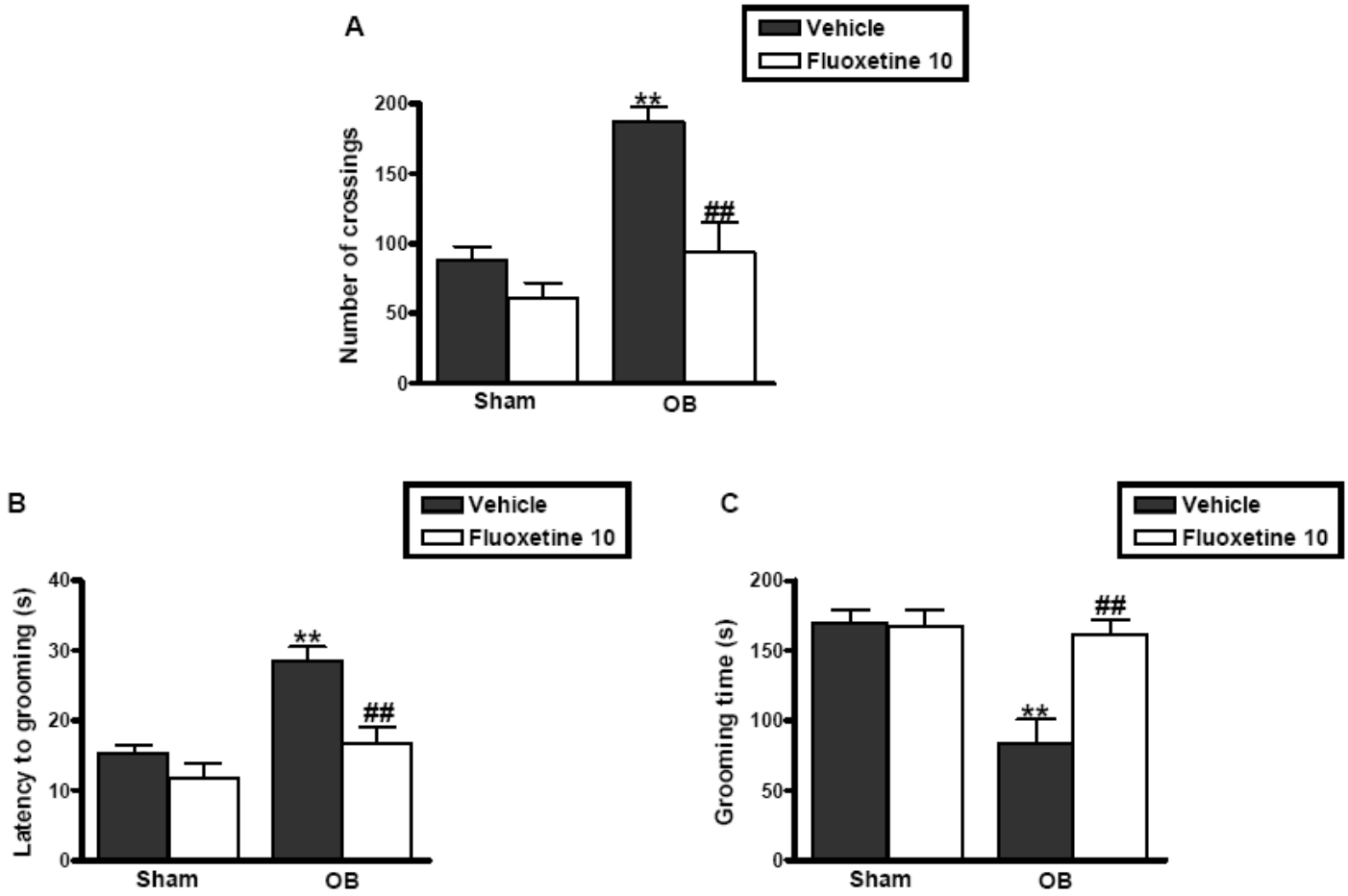

A
Vehicle
Fluoxetine 10
Number of crossings
200
150
100
50
0
**
##
Sham
OB
B
Vehicle
Fluoxetine 10
Latency to grooming (s)
40
30
20
10
0
**
##
Sham
OB
C
Vehicle
Fluoxetine 10
Grooming time (s)
200
150
100
50
0
**
##
Sham
OB

Figure 3.

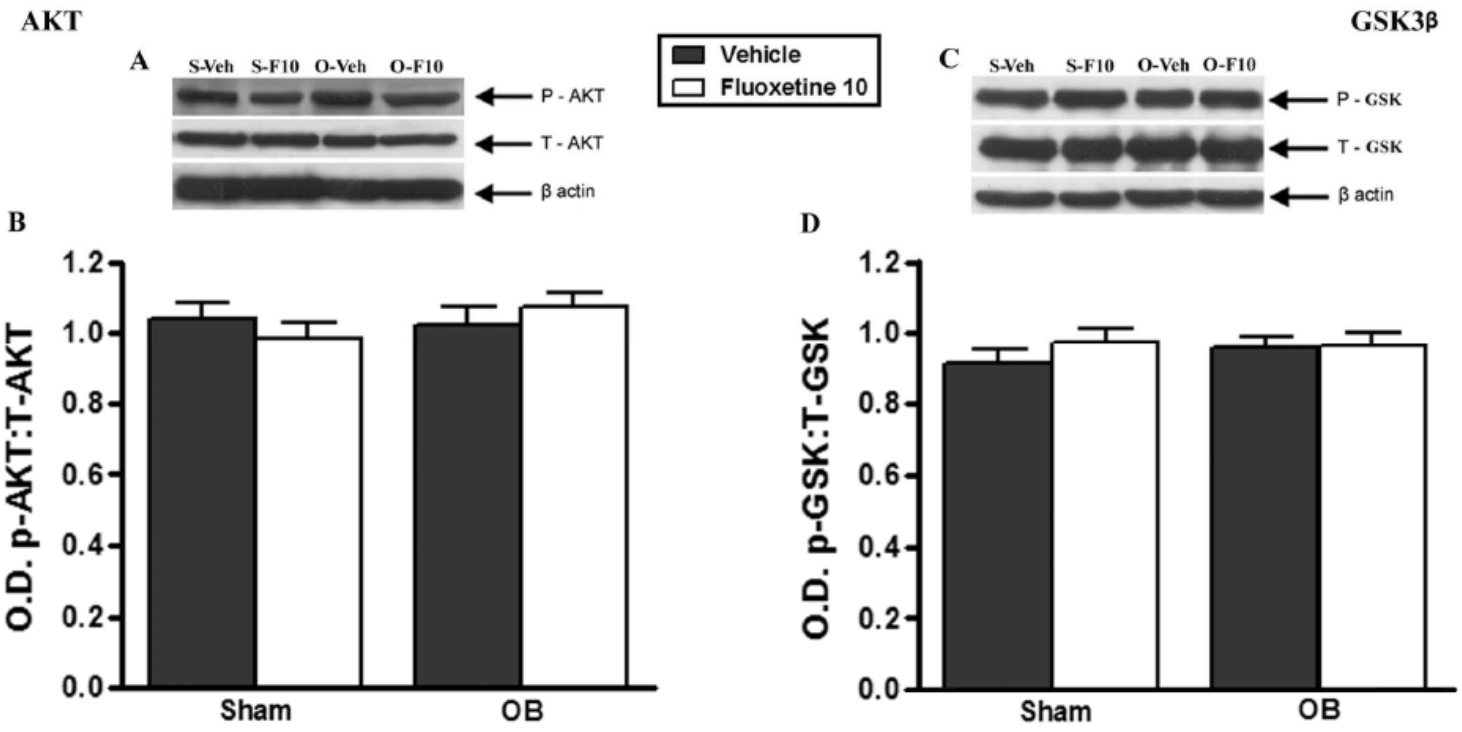

AKT
GSK3β
A
S-Veh S-F10 O-Veh O-F10
P - AKT
T - AKT
β actin
Vehicle
Fluoxetine 10
C
S-Veh S-F10 O-Veh O-F10
P - GSK
T - GSK
β actin
B
O.D. p-AKT:T-AKT
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
OB
D
O.D. p-GSK:T-GSK
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
OB

Figure 4.

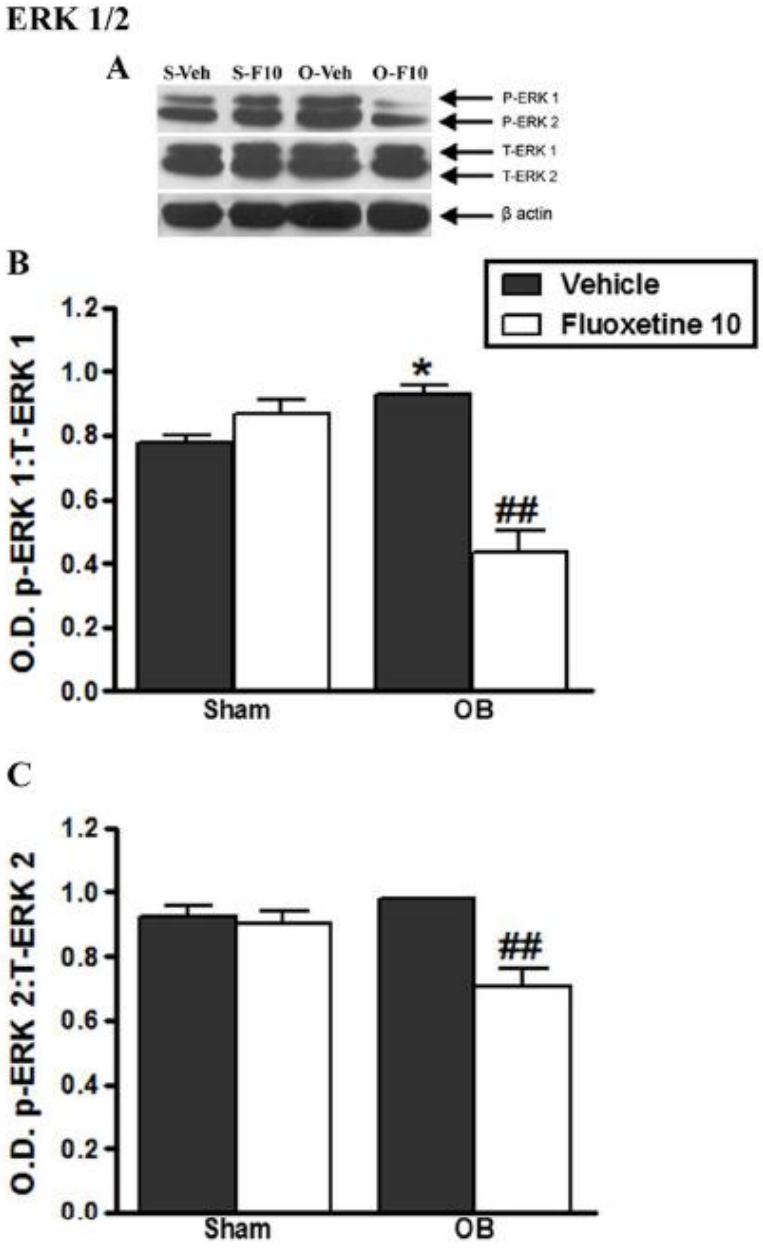
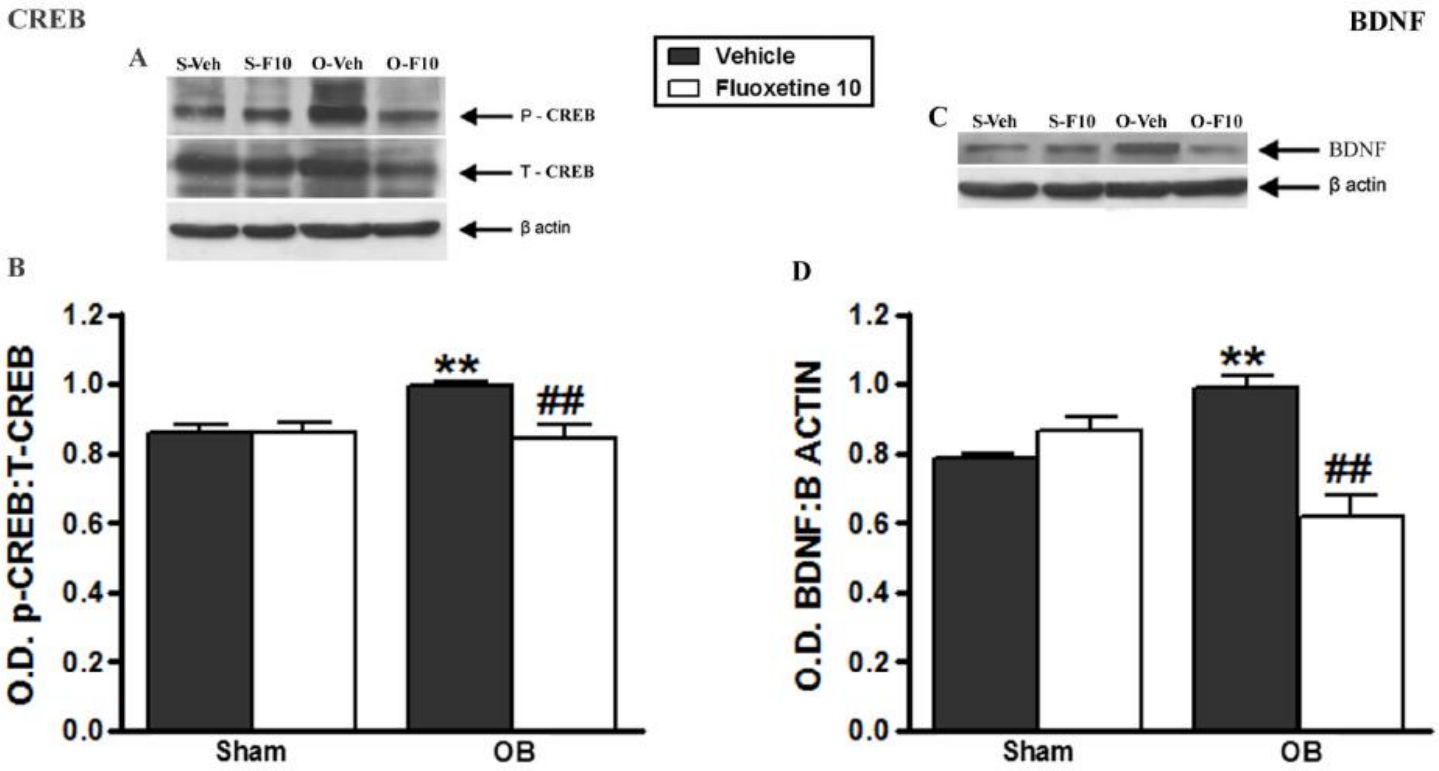

ERK 1/2
A
S-Veh
S-F10
O-Veh
O-F10
P-ERK 1
P-ERK 2
T-ERK 1
T-ERK 2
β actin
B
Vehicle
Fluoxetine 10
O.D. p-ERK 1:T-ERK 1
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
##
Sham
OB
C
O.D. p-ERK 2:T-ERK 2
1.2
1.0
0.8
0.6
0.4
0.2
0.0
##
Sham
OB

Figure 5.


CREB
BDNF
A
S-Veh
S-F10
O-Veh
O-F10
P - CREB
T - CREB
β actin
Vehicle
Fluoxetine 10
C
S-Veh
S-F10
O-Veh
O-F10
BDNF
β actin
B
O.D. p-CREB:T-CREB
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
##
Sham
OB
D
O.D. BDNF:B ACTIN
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
##
Sham
OB

5.3. Manuscrito 3

Participation of NMDA receptors and L-arginine-nitric oxide-cyclic guanosine monophosphate pathway in the antidepressant-like action of the ethanolic extract from *Tabebuia avellanedae* in mice

Andiara E Freitas [a], Morgana Moretti [a], Josiane Budni [a], Grasiela O Balen [a], Sinara C Fernandes [a], Patricia O Veronezi [b], Melina Heller[b], Gustavo A Micke[b], Moacir G Pizzolatti [b], Ana Lúcia S. Rodrigues [a,*]

[a]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário – Trindade - 88040-900, Florianópolis-SC, Brazil

[b]Departamento de Química, Centro de Ciências Físicas e Matemáticas, Universidade Federal de Santa Catarina, Campus Universitário – Trindade - 88040-900, Florianópolis-SC, Brazil

* Corresponding author. Tel.: +55 (48) 3721-5043; fax: +55 (48) 3721-9672.

E-mail address: analucia@mbox1.ufsc.br or ana.rodrigues@pq.cnpq.br

Abstract

Objectives This study was aimed to investigate the contribution of NMDA receptors and L-arginine-NO-cGMP pathway to the antidepressant-like action of the ethanolic extract from *Tabebuia avellanedae* in the tail suspension test (TST).

Methods Immobility time in the TST and locomotion in the open-field test were recorded for a 6-min period in mice.

Key findings The anti-immobility effect of the extract (30 mg/kg, p.o.) was prevented by pre-treatment of mice with NMDA (0.1 pmol/site, i.c.v.), L-arginine (750 mg/kg, i.p., a substrate for nitric oxide synthase) and sildenafil (5 mg/kg, i.p., a phosphodiesterase 5 inhibitor). In addition, the combination of MK-801 (0.01 mg/kg, p.o., a noncompetitive NMDA-receptor antagonist), 7-nitroindazole (25 mg/kg, i.p., a neuronal NO synthase inhibitor) and 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) (30 pmol/site i.c.v., a soluble guanylate cyclase inhibitor) with a sub-effective dose of extract (1 mg/kg, p.o.) produced a synergistic antidepressant-like effect in the TST, without causing significant alterations in locomotor activity.

Conclusions Results suggest that the antidepressant-like effect of the extract from *T. avellanedae* in the TST is dependent on a blockade of NMDA receptor activation and inhibition of NO-cGMP synthesis, significantly extending literature data about the antidepressant-like action of this plant and further reinforcing the pivotal role of these targets in the mechanism of action of antidepressant agents.

Keywords: L-arginine-nitric oxide-cGMP pathway; depression; NMDA receptors; *Tabebuia avellanedae*; tail suspension test

Abbreviations ANOVA, analysis of variance; cGMP, cyclic guanosine 3'5'-monophosphate; DMSO, dimethylsulfoxide, FST, forced swimming test; NO, nitric oxide; NOS, nitric oxide synthase; NMDA, N-methyl-D-aspartate; ODQ, 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one, SNRIs, serotonin and noradrenaline reuptake inhibitors; SSRIs, selective serotonin reuptake inhibitor; 5-HT, serotonin; sGC, soluble guanylate cyclase; TST, tail suspension test.

Introduction

Depression is a common mental disorder associated with high rates of suicide, severe functional impairment, high rates of comorbid mental disorders, intensive use of treatment, and high costs to society[1,2]. According to projections from the World Health Organization, depression will be the second leading cause of disability in the developed world by 2020[3].

Despite the advances in the treatment of depression with selective serotonin reuptake inhibitors (SSRIs) and serotonin and norepinephrine reuptake inhibitors (SNRIs), the major drawbacks of the conventional antidepressant therapy are related to its relatively low efficacy and side effects[4]. Therefore, considerable efforts are invested in the search for better drugs and even combined treatments approaches for the management of depression.

Tabebuia avellanedae Lorentz ex Griseb (Bignoniaceae) is a tree native to tropical rain forests in the northeast of Brazil commonly known as "pau d'arco" or "ipê-roxo". Recently a study by Freitas et al.[5] demonstrated that the administration of the ethanolic extract from barks of *T. avellanedae* exerts an antidepressant-like effect in the tail suspension test (TST), a behavioral test used to assess the efficacy of antidepressant compounds[6]. Additionally, the antidepressant-like action of this extract was reported to be mediated by an activation of the monoaminergic systems. Furthermore, the extract from *T. avellanedae* produced a synergistic antidepressant-like effect when combined with conventional antidepressants[5].

Recent evidences have shown that, besides the monoaminergic systems, several other targets have been implicated in the pathogenesis of depressive disorders. Special emphasis has been given to the N-methyl-D-aspartate (NMDA) receptors and the L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate pathway[7,8]. Additionally,

several biochemical[9], electrophysiological[10] and behavioral[11] studies have shown that increased monoaminergic neurotransmission by treatment with antidepressants is associated with a reduction of either NMDA receptor function or NO synthesis[12,13].

Glutamatergic function is altered in mood disorders and evidence from literature has shown that antidepressants may exert its action by blocking NMDA receptors[7,14]. In addition, glutamatergic modulators, including NMDA receptor antagonists, exhibited antidepressant-like actions in several behavioral paradigms, such as inescapable stress[15], chronic mild stress[16], forced swimming test (FST)[17]. In addition, NMDA receptor antagonists potentiate the antidepressant-like action of serotonergic antidepressants in the FST[18,19]. Riluzole, an antiglutamatergic agent that is believed to act by suppressing presynaptic conduction of glutamatergic neurons and increasing glutamate uptake through the astrocytes showed to be effective in monotherapy and as an augmenting agent in the treatment of refractory depressive subjects[20]. In addition, ketamine, a non-competitive NMDA receptor antagonist, produced a rapid and persistent antidepressant effect in depressed patients[21,22].

Calcium influx, via NMDA receptors, induces the activation of the enzyme nitric oxide (NO) synthase (NOS). The activated NOS then converts L-arginine to NO and L-citrulline[23]. NO, a messenger molecule in the brain has been implicated in pathophysiology of depression[8,24]. In neurons, the best characterized target for NO is soluble guanylate cyclase (sGC) which is activated by NO producing the intracellular messenger cyclic guanosine 3'5'-monophosphate (cGMP)[23].

Several studies have been demonstrated that NOS inhibitors produce anxiolytic and antidepressant-like actions in a variety of animal paradigms[8,24]. In agreement, Harkin and co-workers showed that the administration of NOS inhibitors cause an increase in the effect of 5-HT (serotonin) reuptake inhibitors in the FST[25].

Additionally, it was reported that chronic mild stress change morphology of neurons in the rat hippocampus and that fluoxetine can renormalize those neurons via the inhibition of NOS over-expression[26]. Furthermore, clinical evidence have shown that plasma nitrate and nitrite (NOx) rates and platelet NOS activity are both decreased in depression[27] and that the treatment with antidepressants significantly increase plasma NOx levels[28].

Considering that: a) NMDA receptors and NO-cGMP pathway are involved in the pathogenesis of depression[7,8]; b) the importance of these molecular targets for the efficacy of antidepressants[18,19]; c) the relationship between the monoaminergic systems and the blockade of the NMDA receptors and the modulation of NO synthesis[7,13]; d) the antidepressant-like effect of the ethanolic extract from *T. avellanedae* in the TST has been reported to be dependent on the modulation of the monoaminergic systems[5], but its mechanisms of action still remain to be established; the aim of the present study was to investigate the participation of the NMDA receptors and L-arginine-NO-cGMP pathway in the antidepressant-like effect of the ethanolic extract from *Tabebuia avellanedae* in the TST.

Material and Methods

Plant material and preparation of the ethanolic extract from *T. avellanedae*

T. avellanedae barks were provided by Chamel Indústria e Comércio de Produtos Naturais Ltda (Campo Largo, Brazil), lot 4753. The identification was performed by botanist Elide Pereira dos Santos and a voucher specimen has been deposited at the Herbarium of the Department of Botany at the Universidade Federal do Paraná (UFPR), Brazil. Dried and powdered barks (5 kg) were extracted three times by

maceration with 95% ethanol for 7 days at room temperature. The combined ethanolic extract was filtered, the solvent evaporated under reduced pressure (40-50°C) and lyophilized to give a red-brown solid (919.2 g).

Phytochemical analyses of the bark ethanolic extract from *T. avellanedae*

The analyses of the bark ethanolic extract from *Tabebuia avellanedae* was performed in a capillary electrophoresis system (CE) (HP3DCE, Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector set a 200 nm. The measurements were conducted at 25 °C in an uncoated fused-silica capillary (48.5 cm×50 μm I.D.×375 μm O.D.) obtained from Polymicro (Phoenix, AZ, USA). In the first conditioning, the capillary was washed for 30 min with sodium hydroxide 1.0 M followed deionized water for 30 min. Between runs the capillary was rinsed for 5 min with running electrolyte (sodium tetraborate 20 mmol L-1 and methanol 10%, pH 9.0) Standard solutions and samples were introduced from the inlet capillary extremity and injected hydrodynamically at 50 mbar (50 mbar=4996.2 Pa) for 6 s. The applied separation voltage was 30 kV, positive polarity in the injection side. Caffeic acid (100 mg L-1 was utilized as internal standard and detection at 330 nm. Data acquisition and treatment were performed with HP Chemstation software.

Sample preparation: 0,5299 g of the ethanolic extract was solubilized into 10 mL of the methanol:water 50% (v/v).

Animals

Adult female Swiss mice (30-40 g) were maintained at constant room temperature (20-22°C) with free access to water and food, under a 12:12h light:dark cycle (lights on at 07:00 h). The cages were placed in the experimental room 24 h before the test for acclimatization. All manipulations were carried out between 9:00 and 17:00 h, with each animal used only once (N=8-9 animals per group). The procedures in

this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the local Ethics Committee. All efforts were made to minimize animal suffering and the number of animals used in the experiments

Drugs and treatment

The following drugs were used: L-arginine, NMDA (N-methyl-D-aspartate), (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) (ODQ), 7-nitroindazole (Sigma Chemical Co, USA), MK-801 (RBI, Boston, MA, USA) and sildenafil (Pfizer). All drugs were dissolved in saline, except ODQ which was dissolved in saline with 1% DMSO and 7-nitroindazole that was dissolved in saline with few drops of Tween 80. All drugs were administered by intraperitoneal (i.p.) route in a constant volume of 10 ml/kg body weight, except NMDA and ODQ which were administered by intracerebroventricular (i.c.v.) route. Appropriate vehicle-treated groups were also assessed simultaneously.

I.c.v. administration was performed under ether anesthesia as previously described[29]. Briefly, a 0.4 mm external diameter hypodermic needle attached to a cannula, which was linked to a 25 μl Hamilton syringe, was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse. A volume of 5 μl was then administered in the left lateral ventricle. The injection was given over 30 s, and the needle remained in place for another 30 s in order to avoid the reflux of the substances injected. The injection site was 1 mm to the right or left from the mid-point on a line drawn through to the anterior base of the ears. To ascertain that the drugs were administered exactly into the cerebral ventricle, the brains were dissected and examined macroscopically after the test.

The extract from *T. avellanedae* (1-30 mg/kg) was dissolved in distilled water with 5% Tween 80 and was administered acutely by oral route (p.o.) by gavage 60 min before the TST or the open-field test. The dissolution of the extract was freshly done from the liophylized power immediately before its administration. A control group received distilled water with 5% Tween 80 as vehicle. All the animals were fasted for 120 min before the oral treatment.

In the experiments designed to study whether the antidepressant-like effect of the extract in the TST is mediated through the inhibition of NMDA receptors, mice were pretreated with vehicle or extract (30 mg/kg, p.o.) and 30 min later they received saline or NMDA (0.1 pmol/site, i.c.v.). Sixty minutes later, the TST or the open-field test were carried out.

To investigate a possible synergistic effect of the extract with a NMDA receptor antagonist, animals received by p.o. route a subeffective dose of MK-801 (0.01 mg/kg, p.o., a noncompetitive NMDA-receptor antagonist) or saline and immediately after, a sub-effective dose of the extract (1 mg/kg, p.o.) or vehicle were administered. Sixty minutes later, the TST or the open-field test were carried out.

In a separate series of experiments, the involvement of the L-arginine-nitric oxide pathway in the anti-immobility actions of the extract in the TST was verified. To this end, mice were pre-treated with L-arginine, a precursor of NO (750 mg/kg, i.p., a dose that produces no effect in the TST) or saline and after 30 min they received extract (30 mg/kg, p.o.) or vehicle injection before being tested in the TST after 60 min.

To study the effect of the combined administration of sub-effective doses of the extract (1 mg/kg, p.o.) with sub-effective doses of 7-nitroindazole (25 mg/kg, i.p., a specific neuronal NO synthase inhibitor) or ODQ (30 pmol/site i.c.v., a specific soluble

guanylate cyclase inhibitor), extract or vehicle was administered 30 min before of the drugs and 30 min later the animals were tested in the TST.

We also assessed the role of cyclic GMP (cGMP) in the antidepressant action of the extract. To this end, mice received an injection of sildenafil (5 mg/kg, i.p., a phosphodiesterase (PDE) inhibitor), or saline 30 min before the extract (30 mg/kg, p.o.), and 60 min later, the TST or the open-field test were carried out.

The doses of the drugs used were chosen on the basis of literature and are previously reported not to increase locomotor activity[17,24,29,30]. The dose-response curve of the extract from *T. avellanedae* in the TST was established in a work previously performed by our group[5]. The doses of the extract used in the present study were selected on the basis of these experiments.

Tail suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method described by Steru et al.[6]. Mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was registered during a 6-min period[5,31,32,33].

Open-field test

To assess the effects of the extract from *T. avellanedae* on locomotor activity, mice were evaluated in the open-field paradigm as previously described[34]. Animals were individually placed in a wooden box (40×60×50 cm) with the floor divided into 12 rectangles. The number of squares crossed with all paws (crossing) was counted in a 6

min session. The apparatus were cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

Statistical analysis

Comparisons between experimental and control groups were performed by two-way ANOVA followed by Tukey's HSD test when appropriate. $P<0.05$ was considered significant.

Results

Phytochemical analyses of the ethanolic extract from *T. avellanedae*

Fig. 1 shows the electropherogram of a sample of the bark ethanolic extract from *T. avellanedae*. The analysis of the electropherogram, which has signals at migration time in the range of 2,1-6,5 min, allowed us to identify p-hydroxybenzoic acid (1), anisic acid (2), veratric acid (3) and caffeic acid (4).

Involvement of NMDA receptors in the antidepressant-like effect of the ethanolic extract from *T. avellanedae* in the TST

The results depicted in Fig. 2A show that the pre-treatment of mice with NMDA (0.1 pmol/site, i.c.v.) prevented the antidepressant-like effect of the extract (30 mg/kg, p.o.) in the TST. The two-way ANOVA revealed significant differences of NMDA pre-treatment [$F(1,30)=18.44$, $P<0.01$] and NMDA pre-treatment x extract treatment interaction [$F(1,30)=16.46$, $P<0.01$], but not of extract treatment [$F(1,30)=0.72$, $P=0.40$]. Post hoc analyses indicated that the pre-treatment with NMDA blocked the decrease in immobility time produced by the extract in the TST. The administration of

NMDA alone or in combination with the extract did not affect the ambulation in the open-field (Fig. 2B). The two-way ANOVA revealed no differences for NMDA pre-treatment [$F(1,29)=3.60$, $P=0.07$], extract treatment [$F(1,29)=0.81$, $P=0.38$] and NMDA pre-treatment x extract treatment interaction [$F(1,29)=0.02$, $P=0.90$].

Fig. 2C illustrates the effect of the administration of sub-effective doses of MK-801 (0.01 mg/kg, p.o, a noncompetitive NMDA-receptor antagonist) and extract (1 mg/kg, p.o.) in the TST. The two-way ANOVA revealed significant differences of MK-801 treatment [$F(1,30)=14.20$, $P<0.01$], extract treatment [$F(1,30)=6.23$, $P<0.01$], and MK-801 x extract treatment interaction [$F(1,30)=14.31$, $P<0.01$]. Post hoc analyses indicated that the pre-treatment with a sub-effective dose of the extract (1 mg/kg, p.o.) produced a synergistic effect with MK-801. However, Fig. 2D shows that the co-administration of MK-801 and extract produced no effect in the open-field test. A two-way ANOVA revealed no differences for extract treatment [$F(1,29)=0.48$, $P=0.49$] and MK-801 x extract treatment interaction [$F(1,29)=1.12$, $P=0.30$], but showed a significant effect for MK-801 treatment [$F(1,29)=5.59$, $P<0.05$].

Effect of pre-treatment of mice with L-arginine on the extract from *T. avellanedae*-induced anti-immobility effect in the TST

The results illustrated in Fig. 3A show that the pre-treatment of mice with L-arginine (750 mg/kg, i.p., an NO precursor) prevented the antidepressant-like effect of the extract (30 mg/kg, p.o.) in the TST. The two-way ANOVA revealed significant differences of L-arginine pre-treatment [$F(1,30)=6.51$, $P<0.01$], extract treatment [$F(1,30)=17.16$, $P<0.01$] and L-arginine pre-treatment x extract treatment interaction [$F(1,30)=12.91$, $P<0.01$]. Post hoc analyses indicated that the anti-immobility effect of the extract was completely prevented by pre-treatment of animals with L-arginine. Fig.

3B shows that the administration of L-arginine and extract did not influence the locomotor activity of mice. The two-way ANOVA revealed no differences for L-arginine pre-treatment [$F(1,30)=3.20$, $P=0.08$], extract treatment [$F(1,30)=0.03$, $P=0.86$] and L-arginine pre-treatment x extract treatment interaction [$F(1,30)=1.18$, $P=0.30$].

Effect of combined administration of the extract from *T. avellanedae* with NOS and soluble glanylate cyclase inhibitors in the TST

The results depicted in Fig. 4A show that the combined of a sub-effective dose of the extract (1 mg/kg, p.o.) and a sub-effective dose of 7-nitroindazole (25 mg/kg, i.p., a neuronal NOS inhibitor), reduced the immobility time of mice submitted to the TST. The two-way ANOVA revealed significant differences of 7-nitroindazole treatment [$F(1,30)=14.65$, $P<0.01$], extract treatment [$F(1,30)=10.14$, $P<0.01$] and 7-nitroindazole x extract treatment interaction [$F(1,30)=11.65$, $P<0.01$]. Post hoc analyses revealed a synergistic antidepressant-like effect induced by 7-nitroindazole when co-administered with the extract in the TST. Fig. 4B illustrates that the administration of 7-nitroindazole in combination with the extract did not affect animal ambulation. A two-way ANOVA revealed no differences for extract treatment [$F(1,29)=0.02$, $P=0.88$] and 7-nitroindazole x extract treatment interaction [$F(1,29)=0.01$, $P=0.99$], but showed a significant effect for 7-nitroindazole treatment [$F(1,29)=5.27$, $P<0.05$].

Fig. 4C shows that the administration of a sub-effective dose of ODQ (30 pmol/site i.c.v., a soluble guanylate cyclase inhibitor) exhibited an antidepressant-like effect when combined with a sub-effective dose of the extract (1 mg/kg, p.o.). The two-way ANOVA revealed significant differences of ODQ treatment [$F(1,30)=13.35$, $P<0.01$], extract treatment [$F(1,30)=35.17$, $P<0.01$] and ODQ x extract treatment interaction [$F(1,30)=39.84$, $P<0.01$]. Post hoc analyses indicated that the pre-treatment

with a sub-effective dose of the extract (1 mg/kg, p.o.) produced a synergistic effect with ODQ. In addition, the administration of ODQ (30 pmol/site i.c.v.) alone or in combination with the extract did not significantly affect the ambulation in the open-field (Fig. 4D). A two-way ANOVA revealed no differences for ODQ treatment [$F(1,30)=3.28$, $P=0.08$], extract treatment [$F(1,30)=0.14$, $P=0.71$] and ODQ x extract treatment interaction [$F(1,30)=2.34$, $P=0.14$].

Effects of pretreatment with sildenafil in the antidepressant-like action of the extract from *T. avellanedae* in the TST

Fig. 5A shows that the anti-immobility effect of the extract (30 mg/kg, p.o.) was completely prevented by pre-treatment of animals with sildenafil (5 mg/kg, i.p., a PDE_5 inhibitor). The two-way ANOVA revealed significant differences of sildenafil pre-treatment [$F(1,29)=5.66$, $P<0.05$], extract treatment [$F(1,29)=26.57$, $P<0.01$] and sildenafil pre-treatment x extract treatment interaction [$F(1,29)=18.93$, $P<0.01$]. Post hoc analyses indicated that the pre-treatment of mice with sildenafil prevented the decrease in immobility time in the TST produced by the administration of the extract. The administration of sildenafil (5 mg/kg, i.p.) alone or in combination with the extract did not affect the ambulation of mice (Fig. 5B). A two-way ANOVA revealed no differences for sildenafil pre-treatment [$F(1,30)=0.01$, $P=0.98$], extract treatment [$F(1,30)=0.02$, $P=0.88$] and sildenafil pre-treatment x extract treatment interaction [$F(1,30)=0.65$, $P=0.43$].

Discussion

The preset work further contributes to the understanding of the mechanisms underlying the antidepressant-like action of the ethanolic extract from *Tabebuia*

avellanedae in the TST, which was recently demonstrated, by our group, to be dependent on an interaction with the monoaminergic systems[5].

The present study shows that the extract from *T. avellanedae* given systemically (by p.o. route) is able to produce an anti-immobility effect through the modulation of either NMDA receptors or L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) signaling pathway. In addition, this study extends literature by reinforcing the pivotal role of inhibition of NMDA receptors and NO-cGMP synthesis in the mechanism underlying the effects of antidepressant agents.

The TST is a behavioral tool widely used for screening antidepressant activity of different classes of drugs[6,35]. This test is based on the observation that animals, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable stressful situation. When antidepressant treatments are given prior to the tests, the subjects will actively persist engaging in escape-directed behavior for longer periods of time than after vehicle treatment[35]. In this study, a sub-effective (1 mg/kg) and an effective (30 mg/kg) dose of the extract from *T. avellanedae* were chosen on the basis of a previous study performed by our group[5]. Noteworthy, these doses did not change the ambulatory activity of mice. It is relevant to mention that this study was performed in female mice, since several studies have shown that the prevalence of depression is about two fold higher in women than in men[36].

The glutamatergic system plays a significant role in depressive disorders[7,37]. The implication of NMDA inhibition in the mechanism of action of conventional antidepressants and putative antidepressant agents has been suggested by several studies which show that the administration of NMDA is able to reverse the antidepressant-like effects of these compounds[9,13,29]. In the present study, the pre-treatment of mice with NMDA blocked the antidepressant-like effect of the extract from *T. avellanedae* in the

TST, also suggesting that the antidepressant-like effect of the extract may be dependent on the inhibition of NMDA receptor activation. Further reinforcing this notion, the co-administration of mice with sub-effective doses of the extract from *T. avellanedae* and MK-801, a noncompetitive NMDA receptor antagonist, produced a synergistic antidepressant-like effect in the mouse TST. The reduction of locomotor activity observed in the group of mice that received MK-801 and the extract did not account for the antidepressant-like effect induced by the combined administration of them. Noteworthy, MK-801, as well as other competitive and uncompetitive NMDA antagonists, exhibit antidepressant-like actions in the FST[7,15] and TST[30]. Our results are consistent with the fact that compounds that reduce transmission at NMDA receptors exert antidepressant-like actions[17,29,30]. However, our results do not allow us to conclude about the mechanism by which the extract interacts with the NMDA receptor. Preclinical and clinical observations have shown that antidepressant drugs reduce NMDA binding, expression and function[7,38]. Additionally, several highly selective NR2B antagonists have been reported to improve depressive symptoms in patients resistant to biogenic-amine-based agents[39].

This study also evaluated the involvement of the L-arginine-NO-cGMP signaling pathway in the antidepressant-like action of the extract from *T. avellanedae*. It has become generally accepted that NO plays a significant neuromodulatory role in the nervous system and the pharmacological manipulation of NO-cGMP signaling pathway may constitute a novel therapeutic approach for the management of depression[8,24].

In the present study we demonstrate that pretreatment with the NOS substrate, L-arginine, significantly prevented the anti-immobility effect of the extract from *T. avellanedae*. Similarly, in another study, the antidepressant effects of imipramine were also blocked by pretreatment with L-arginine and contrary to this, NOS inhibitor, N^G-

nitro-Larginine augmented the behavioral effect of imipramine or fluoxetine in the FST[25]. Thus, our result indicated that the inhibition of NO synthesis may underlie the reduction of the immobility time in the TST elicited by the extract from *T. avellanedae*.

Supporting the notion that the effect of extract from *T. avellanedae* in the TST is, at least in part, due to an inhibition of NO synthesis, in our study we also demonstrated that the co-treatment of 7-nitroindazole (a specific neuronal NO synthase inhibitor) or ODQ (an inhibitor of soluble guanylate cyclase) with a sub-effective dose of the extract produced a synergistic antidepressant-like effect in the TST. Considering that NO activates guanylate cyclase, that generates cGMP, which mediates many of the effects of NO[23], our results also provides evidence that the antidepressant-like effect of the extract may be mediated through the reduction of cGMP levels, probably as a consequence of the reduction of NO synthesis.

Taken together, these results are in accordance with the finding that NOS inhibitors, depending on their concentrations, exert antidepressant-like effects in a variety of animal models of depression[8,24,40], and are able to enhance the activity of antidepressants that act through serotonergic mechanisms[25]. In addition, a study by Krass and co-workers showed that the treatment with imipramine and venlafaxine decreased the nitrite plus nitrate content in brain, and this mechanism appears to be important for the antidepressant action of these drugs[12].

Another piece of evidence that the extract from *T. avellanedae* exerts its effect in the TST by decreasing cGMP levels is given by the reversal of the anti-immobility effect of the extract from *T. avellanedae* by the PDE5 inhibitor sildenafil. PDE5 catalyses the hydrolysis of the second messenger cGMP and is particularly expressed in the cerebellum and hippocampus[41]. Therefore, are results are in agreement with the

notion that the inhibition of cGMP synthesis may be an important target to promote antidepressant effects.

Conclusions

In conclusion, the present study first indicates, to our knowledge, that *T. avellanedae* produces an antidepressant-like action in the TST by modulation of NMDA receptors and L-arginine-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) signaling cascade. Taken together, our results support evidence that these pathways could be relevant to the antidepressant effect of this plant.

Funding

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Declarations

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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Legend to the figures:

Fig. 1. Eletrocpherogram of the ethanolic extract from *Tabebuia avellanedae*. p-hydroxybenzoic acid (1), anisic acid (2), veratric acid (3) and caffeic acid (4).

Fig. 2. Effect of the pre-treatment of mice with NMDA (0.1 pmol/site, i.c.v.) on the anti-immobility effect of the ethanolic extract from *Tabebuia avellanedae* (30 mg/kg, p.o.) in the TST (panel A) and on locomotor activity in the open-field test (panel B). Effect of the combined treatment of mice with the extract from *Tabebuia avellanedae* (1 mg/kg, p.o) and MK-801 (0.01 mg/kg, p.o.) in the TST (panel C) and on the number of crossings in the open-field test (panel D). Each column represents the mean+S.E.M of 8-9 animals. **P<0.01 compared with the vehicle-treated control. ##P<0.01 as compared with the extract alone.

Fig. 3. Effect of the pre-treatment of mice with L-arginine (750 mg/kg, i.p.) on the anti-immobility effect of the ethanolic extract from *Tabebuia avellanedae* (30 mg/kg, p.o.) in the TST (panel A) and on locomotor activity in the open-field test (panel B). Each column represents the mean+S.E.M of 8-9 animals. **P<0.01 compared with the vehicle-treated control. ##P<0.01 as compared with the extract alone.

Fig. 4. Effect of the administration of a subeffective dose of the ethanolic extract from *Tabebuia avellanedae* (1 mg/kg, p.o.) with subeffective doses of 7-nitroindazole (25 mg/kg, i.p.) and ODQ (30 pmol/site i.c.v.) in the TST (panel A and C, respectively) and in the open-field test (panel B and D, respectively). Each column represents the mean+S.E.M of 8-9 animals. **P<0.01 as compared with the vehicle-treated group.

Fig. 5. Effect of the pre-treatment of mice with sildenafil (5 mg/kg, i.p.) on the anti-immobility effect of the ethanolic extract from *Tabebuia avellanedae* (30 mg/kg, p.o.) in the TST (panel A) and on the locomotor activity in the open-field test (panel B). Each column represents the mean+S.E.M of 8-9 animals. **P<0.01 compared with the vehicle-treated control. ##P<0.01 as compared with the extract alone.

Figure 1.

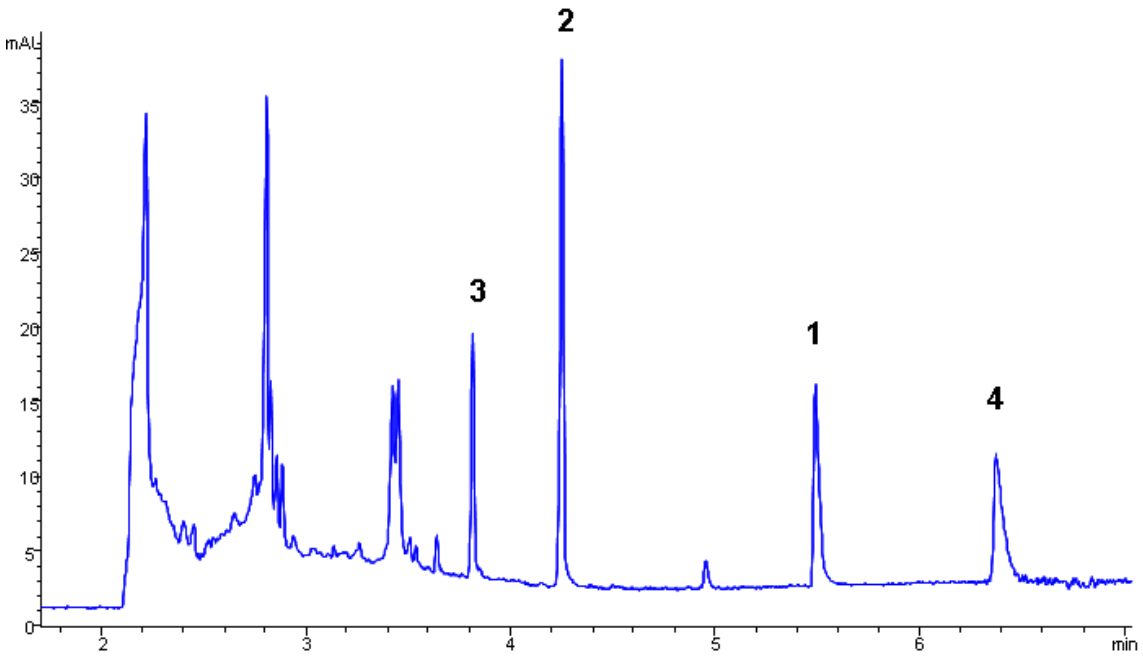


Figure 2.

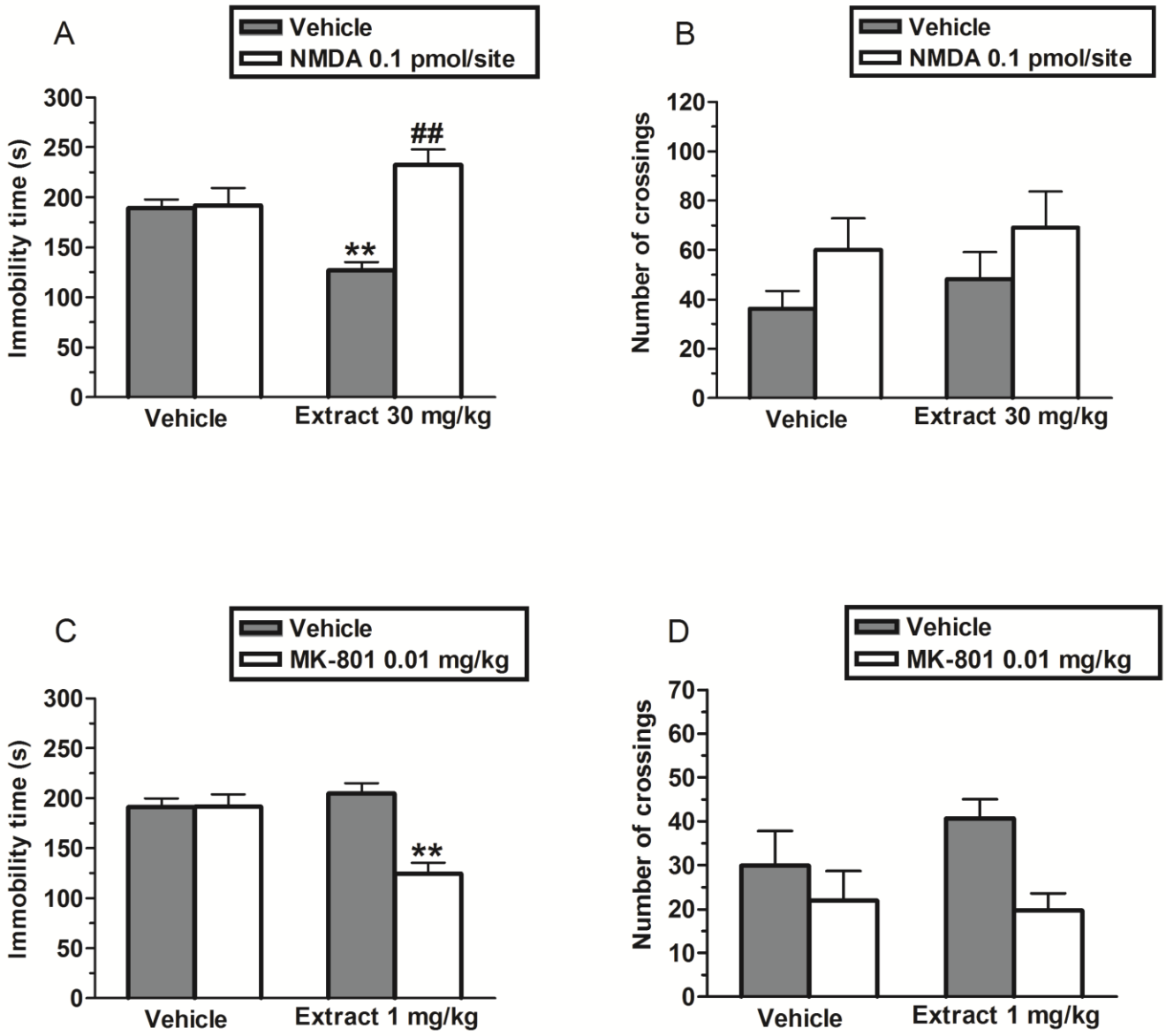

A
Vehicle
NMDA 0.1 pmol/site
Immobility time (s)
300
250
200
150
100
50
0
**
##
Vehicle
Extract 30 mg/kg
B
Vehicle
NMDA 0.1 pmol/site
Number of crossings
120
100
80
60
40
20
0
Vehicle
Extract 30 mg/kg
C
Vehicle
MK-801 0.01 mg/kg
Immobility time (s)
300
250
200
150
100
50
0
**
Vehicle
Extract 1 mg/kg
D
Vehicle
MK-801 0.01 mg/kg
Number of crossings
70
60
50
40
30
20
10
0
Vehicle
Extract 1 mg/kg

Figure 3.

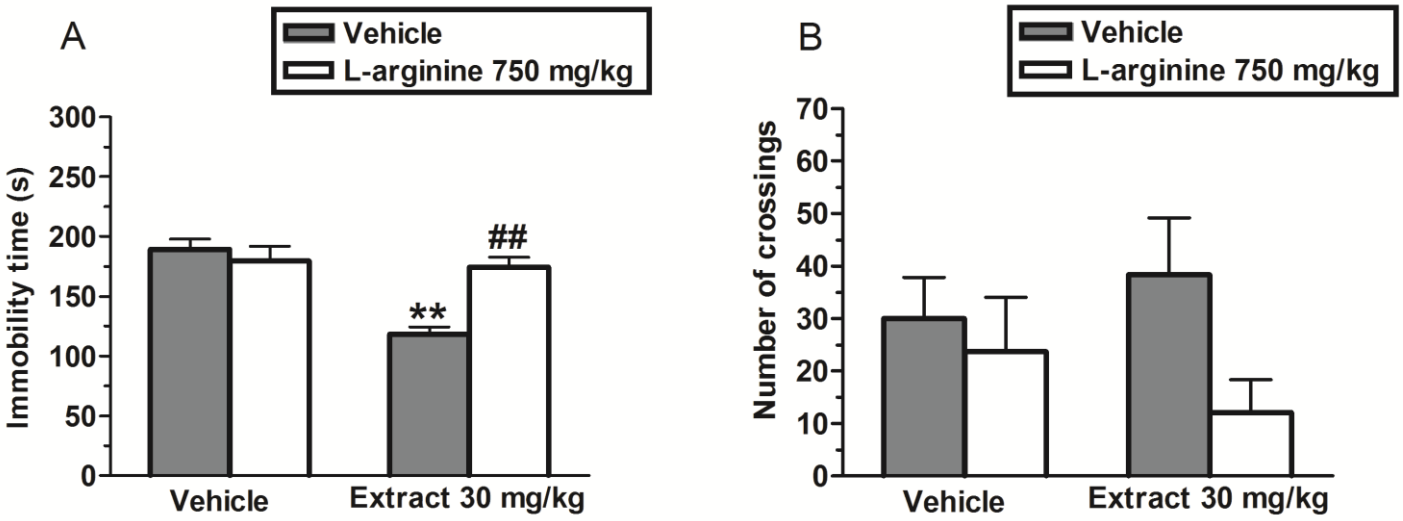

A
Vehicle
L-arginine 750 mg/kg
Immobility time (s)
300
250
200
150
100
50
0
**
##
Vehicle
Extract 30 mg/kg
B
Vehicle
L-arginine 750 mg/kg
Number of crossings
70
60
50
40
30
20
10
0
Vehicle
Extract 30 mg/kg

Figure 4.

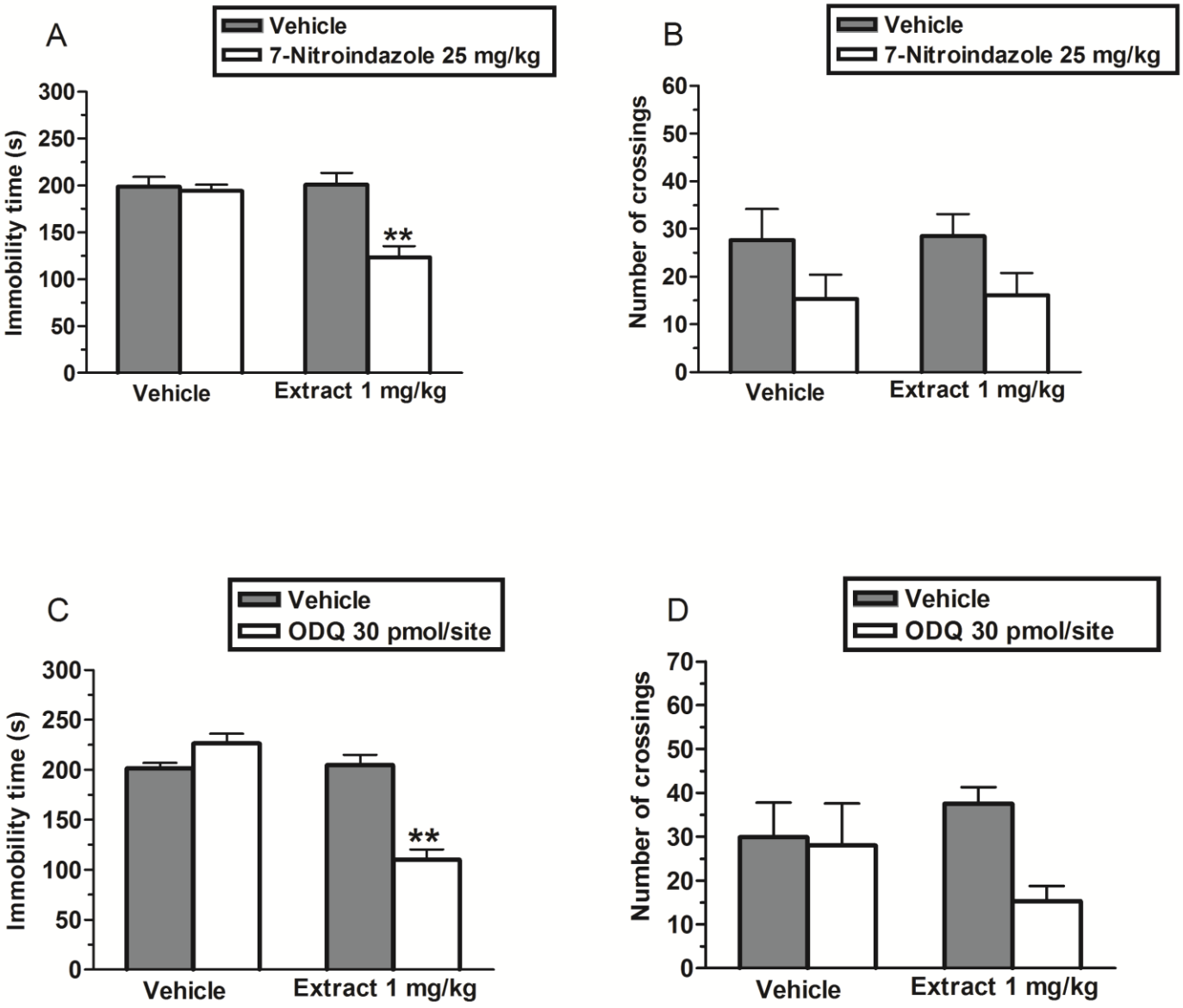
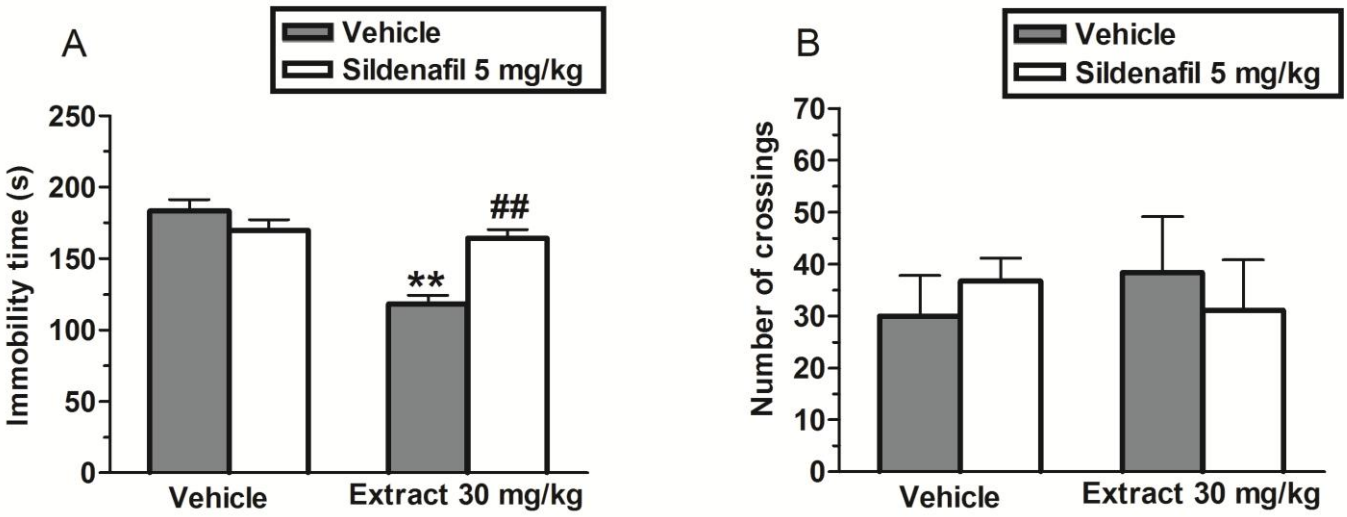

A
Vehicle
7-Nitroindazole 25 mg/kg
Immobility time (s)
300
250
200
150
100
50
0
**
Vehicle
Extract 1 mg/kg
B
Vehicle
7-Nitroindazole 25 mg/kg
Number of crossings
60
50
40
30
20
10
0
Vehicle
Extract 1 mg/kg
C
Vehicle
ODQ 30 pmol/site
Immobility time (s)
300
250
200
150
100
50
0
**
Vehicle
Extract 1 mg/kg
D
Vehicle
ODQ 30 pmol/site
Number of crossings
70
60
50
40
30
20
10
0
Vehicle
Extract 1 mg/kg

Figure 5.


A
Vehicle
Sildenafil 5 mg/kg
Immobility time (s)
250
200
150
100
50
0
**
##
Vehicle
Extract 30 mg/kg
B
Vehicle
Sildenafil 5 mg/kg
Number of crossings
70
60
50
40
30
20
10
0
Vehicle
Extract 30 mg/kg

6. Discussão

Este estudo ampliou evidências farmacológicas e bioquímicas a respeito do efeito tipo-antidepressivo do extrato etanólico de *T. avellanedae*, que recentemente, foi demonstrado pelo nosso grupo ser dependente de sua interação com os sistemas monoaminérgicos (Freitas et al., 2010). No presente estudo, foi demostrado que a administração por via oral durante 14 dias de extrato de *T. avellanedae* produz um significativo efeito do tipo-antidepressivo no TSC – teste preditivo amplamente utilizado para avaliação da ação antidepressiva de compostos (Bourin et al., 2005). Adicionalmente, o extrato de *T. avellanedae* exibiu uma ação antidepressiva em um modelo de indução de depressão bem estabelecido na literatura – a bulbectomia olfatória. O tratamento dos animais bulbectomizados durante 14 dias com extrato de *T. avellanedae* foi efetivo em prevenir a hiperatividade e o comportamento anedônico induzidos pela ablação dos bulbos olfatórios, de maneira semelhante ao antidepressivo fluoxetina. O modelo da BO, padronizado em nosso laboratório reproduziu importantes alterações comportamentais relatadas na literatura, principalmente o aumento da atividade locomotora no TCA e a anedonia no splash teste. O tratamento (durante 14 dias) dos animais BO com o extrato de *T. avellanedae* foi efetivo em reverter a hiperatividade e o comportamento anedônico produzidos pela remoção dos bulbos olfatórios, de maneira semelhante que a fluoxetina o fez. Adicionalemte, o estudo teve por objetivo ampliar o aporte da literatura a respeito dos efeitos da BO sobre a expressão de proteínas relacionadas à sobreviva e morte celular como Akt, GSK-3β, ERK1/2, CREB e BDNF na região hipocampal através da técnica de Western blot. A ablação dos bulbos olfatórios aumentou significativamente os níveis de fosforilação de CREB e ERK1 bem como o imunoconteúdo de BDNF. O tratamento *per se* com extrato (14 dias) produziu um aumento significativo da fosforilação de CREB (Ser^{133}) e GSK-

3β (Ser^{9}). Além disso, foi efetivo em rerverter o aumento de pERK1 e do imunoconteúdo de BDNF, de maneira semelhante à fluoxetina. Adicionalmente, o antidepressivo fluoxetina preveniu o aumento de pCREB e diminuiu significativamente os níveis de pERK2 no grupo BO. Nenhum dos tratamentos, nem a BO causou alguma alteração nos níveis de fosforilação de Akt (Ser^{473}). Nosso conjunto de resultados ampliou significativamente os conhecimentos da literatura sobre alterações comportamentais e bioquímicas induzidas pela BO e elucidou alguns mecanismos envolvidos na ação antidepressiva do extrato de *T. avellanedae* e do antidepressivo fluoxetina, neste modelo.

O TSC – teste preditivo animal para avaliação da ação antidepressiva de compostos, é baseado na observação dos animais que após um período de movimentação inicial, desenvolvem uma postura imóvel quando submetidos a uma situação de stress inescapável (Porsolt et al., 1977; Cryan et al., 2005a). Os antidepressivos clássicos exibem uma redução do tempo de imobilidade dos animais quando submetidos a este teste (Cryan et al., 2005a). Em um estudo anterior, demonstramos que a administração aguda do extrato de *T. avellanedae* produz efeito tipo-antidepressivo no TSC em camundongos (Freitas et al., 2010). No presente estudo, o tratamento durante 14 dias com extrato nas doses de 10 e 30 mg/kg (p.o.) produziu um significativo efeito antidepressivo no TSC em camundongos corroborando com o fato de que medicamentos antidepressivos exibem uma redução no tempo de imobilidade neste teste preditivo (Artaiz et al., 2005).

Além do TSC, há vários modelos animais utilizados para avaliar a eficácia de antidepressivos como o TNF (Cryan et al., 2005b) e o desamparo aprendido (Seligman et al., 1975). A principal crítica destes testes é que a atividade antidepressiva é verificada após administração aguda da substância a ser avaliada, enquanto que na

prática clínica, vários dias ou semanas são necessários para que o efeito terapêutico seja observado (Yan et al., 2010). Uma exceção a esta regra é o modelo da bulbectomia olfatória que é capaz de avaliar o efeito antidepressivo quase que exclusivamente após tratamento crônico e reproduz alterações comportamentais compatíveis ao que é observado nos pacientes deprimidos como disfunções de aprendizado e memória, agitação psicomotora e anedonia (Kelly et al., 1997). Ressalta-se ainda, que os efeitos causados pela BO não podem ser explicados simplismente pela indução de anosmia, uma vez que, a destruição dos receptores sensoriais olfatórios não reproduz os sintomas característicos da BO (Alberts and Friedman, 1972; Saitoh et al., 2006).

O aumento da atividade locomotora no TCA é um parâmetro que confere validação para o procedimento cirúrgico (Kelly et al., 1997) e reflete a agitação psicomotora, um sintoma-chave para diagnóstico de episódios depressivos (American Psychiatry Association, 2000). No presente estudo, a ablação dos bulbos olfatórios causou uma hiperlocomoção consistente com sintomas de depressão agitada que foi significativamente prevenida pelo tratamento (14 days) com extrato 10 e 30 mg/kg (p.o.) e com fluoxetina 10 mg/kg (p.o.). A abilidade do extrato e da fluoxetina em reverterem sintomas de depressão agitada corrobora com vários estudos que mostram que antidepressivos como a fluoxetina (Mar et al., 2002; Roche et al., 2007), amitriptilina e o citalopram são capazes de produzirem este efeito.

Anedonia é um sintoma afetivo caracterizado pela perda de interesse ou satisfação em quase todas as atividades que é passível de ser avaliada pelo splash teste. Neste teste, um aumento do tempo de latência bem como uma diminuição do tempo de *grooming* indica um comportamento depressivo (Kalueff and Tuohimaa, 2004). Nossos resultados estão de acordo com esta linha, uma vez que, a destruição dos bulbos olfatórios induziu uma perda motivacional e do auto-cuidado caracterizado pelo

aumento do tempo de latência e diminuição do tempo de *grooming* observados nos animais BO. Adicionalmente, os tratamentos com extrato 10 e 30 mg/kg (p.o.) e com fluoxetina 10 mg/kg (p.o.) foram efetivos em reverterem este comportamento anedônico estando de acordo com estudos recentes que têm demonstrado que compostos antidepressivos são capazes de prevenirem a indução de anedonia em modelos animais de depressão como o stress de contenção (Hayase, 2011), o stress crônico imprevisível (Li et al., 2011) e o stress crônico moderado (Elizalde et al., 2010).

Considerando que a eficácia de agentes antidepressivos não pode ser explicada somente por seus efeitos no sistema monoaminérgico, e que adaptações moleculares mediadas pela ativação de vias de sinalização podem estar envolvidas nestes efeitos (Fisar and Hroudová, 2010), o presente estudo se propôs a avaliar os efeitos da BO sobre vias de sinalização como a Akt, GSK-3β, ERK-1/2, CREB e BDNF na região hipocampal e a habilidade do tratamento repetido (14 dias) com extrato de *T. avellanedae* e com fluoxetina de alterar esstes parâmetros nos animais do grupo Sham bem como nos BO.

GSK-3 é uma proteína do tipo serina/treonina cinase envolvida em plasticidade sináptica (Peineau et al., 2008), neurogênse (Hur and Zhou, 2010) e comportamento do tipo-antidepressivo (Rosa et al., 2008). Pode ser identificada sob duas isoformas, α e β. GSK-3β tem sido implicada na patogênese de várias doenças neuropsiquiátricas como transtorno bipolar, esquizofrenia e depressão. Esta enzima tem sido proposta como um importante alvo para novos fármacos a serem utilizados nestas doenças (Beaulieu et al., 2009). GSK-3β está constitutivamente ativa, porém é regulada via fosforilação na região N-terminal Ser^9 (Peineau et al., 2008) por cinases como Akt/PKB, proteína cinase A (PKA) e proteína cinase C (PKC) (Doble and Woodgett, 2003) sendo então inibida. Akt/PKB é uma serina/treonina cinase que fosforila e regula a função de proteínas

celulares envolvidas em apoptose, proliferação e neuroplasticidade (Song et al., 2005), incluindo GSK-3β. Akt/PKB contém dois sítios regulatórios de fosforilação, Thr^{308} e Ser^{473} (Nicholson and Anderson, 2002). O envolvimento da Akt e da GSK-3β nos efeitos comportamentais induzidos pela BO não estão bem estabelecidos. No presente estudo, nem o tratamento com fluoxetina nem a ablação dos bulbos olfatórios causou alguma alteração dos níveis de pAkt (Ser^{473}) ou de pGSK-3β (Ser^{9}), entretanto o tratamento com extrato de *T. avellanedae* (30 mg/kg, p.o.), foi capaz de aumentar significativamente os níveis de fosforilação (Ser^{9}) de GSK-3β, o que corrobora com estudos que demonstram que compostos antidepressivos são capazes de aumentar os níveis de fosforilação de GSK-3β (Ser^{9}) na região do córtex cerebral (Li et al., 2004) e do hipocampo (Eom and Jope, 2009), promovendo uma inibição da atividade desta enzima.

ERK1 e ERK2 são membros da família das MAPKs e têm sido envolvidas em sobrevida/morte, crescimento e diferenciação celular (Johnson and Lapadat, 2002; Strnisková et al., 2002; Stork and Schmitt, 2002). ERK1/2 são ativadas pela interação do BDNF a ser receptor TrkB através da via das Ras-Raf-MAPK cinase (MEK1/2)-ERK1/2 induzindo translocação e fosforilação de fatores de transcrição. Quando ativado, o estado fosforilado de ERK1/2 regula crescimento, diferenciação e morte neuronal (Stork and Schmitt, 2002). Evidencias da literatura têm demostrado que a via ERK está envolvida na modulação da depressão (Fumagalli et al., 2005; Todorovic et al., 2009). Adicionalmente, foi relatado um aumento dos níveis de fosforilação de ERK1/2 na região hipocampal após indução de comportamento depressivo pelo stress e que o tratamento crônico com desipramina foi efetivo em prevenir este comportamento e o aumento de pERK1 (Bravo et al., 2009). Neste contexto, nossos resultados mostraram que o aumento dos níveis de fosforilação de ERK1 produzida pela BO foi

significativamente prevenida pelo tratamento (14 dias) com extrato (10 e 30 mg/kg, p.o.) e com fluoxetina (10 mg/kg, p.o.), reforçando a idéia de que a inibição de ERK1/2 produz efeitos antidepressivos (Einat et al., 2003; Galeotti and Ghelardini, 2011). Reforçando esta hipótese, o tratamento dos animais bulbectomizados com fluoxetina diminuiu significativamente os níveis de pERK2 na região hipocampal. Neste sentido, nossos resultados sugerem que a inibição de ERK1/2 hipocampal parece estar envolvida no efeito antidepressivo do extrato de *T. avellanedae* e da fluoxetina nos animais BO.

CREB é um fator de transcrição nuclear que no cérebro está envolvido em neuroplasticidade. Desta maneira, muitos fenômenos fisiológicos incluindo memória, aprendizado, neuroproteção, transmissão sináptica, sobrevida neuronal, diferenciação celular, crescimento axonal podem ser modulados por CREB (Lonze and Ginty, 2002; Stern et al., 2011). Ser^{133} no CREB é um algo bem elucidado para muitas proteínas cinases, incluindo PKA, PKG, PKC, Akt/PKB, MAPKs (via Rsk) (Shaywitz and Greenberg, 1999). Considerando que estas cinases estão envolvidas em vias de diferentes sistemas de transdução, CREB desempenha um importante papel interagindo muitos sinais neuronais.

A administração crônica de antidepressivos up-regula a neurogênese mediada por CREB na região hipocampal (Li et al., 2009). Dados da literatuta têm mostrado que compostos do tipo-antidepressivos induzem um aumento da expressão de CREB na região do giro denteado hipocampal de ratos (Chen et al., 2001; Qi et al., 2008). No presente estudo, o tratamento durante 14 dias com extrato de *T. avellanedae* (10 e 30 mg/kg, p.o.) produziu um aumento significativo dos níveis de pCREB hipocampal em ambos os animais Sham e BO, corroborando com a idéia de que CREB é up-regulado pelo tratamento crônico com antidepressivos e que induz comportamentos do tipo-antidepressivos (Chen et al., 2001; Qi et al., 2008).

Adicionalmente, tem sido proposto que CREB pode ser ativado não somente em resposta a um estímulo de crescimento e sobrevivência, mas também em resposta a estímulos stressores (Kageyama et al., 2010). Nos neurônios, CREB é fosforilado sob condições de hipóxia e stress oxidativo, sugerindo que a ativação de um programa de sbrevivência dependente de CREB em resposta a um insulto pode representar uma forma de defesa celular (Delivoria-Papadopoulos, 2011). De fato, fortes evidências cofirmam o papel de CREB como neuroprotetor (Stern et al., 2011). Nossos resultados mostraram que a remoção dos bulbos olfatórios induziu um aumento significativo dos níveis de fosforilação de CREB em Ser^{133} na região hipocampal. Recentemente foi relatado que o stress psicosocial crônico – um modelo animal de depressão, estimula a atividade transcripcional de CREB e que é prevenida pelo tratamento com antidepressivos como imipramina (Böer et al., 2007), citalopram e fluoxetina (Böer et al., 2010). Interessantemente, em nosso estudo observamos um aumento de pCREB hipocampal nos animais BO, que pode indicar uma resposta adaptativa ao rompimento das conexões neuronais. De maneira semelhante, o estudo de Romeas e colaboradores (2009) demonstraram um aumento de pCREB na região estriatal nos animais bulbectomizados. O aumento dos níveis de fosforilação de CREB induzida pela ablação dos bulbos olfatórios foi prevenida pelo tratamento (14 dias) com fluoxetina (10 mg/kg, p.o.), reforçando a idéia de que CREB pode ser um alvo importante para a indução de comportmento depressivo causado pela BO.

A hipótese neurotrófica da depressão postula que um déficit neurotrófico, especialmente de BDNF hipocampal está envolvido na patogênese desta doença (Duman et al., 1997). No presente estudo, foi observado um aumento dos níveis de BDNF nos animais BO e está de acordo com o estudo de Hellweg e seu grupo (2007), que mostra que a ablação dos bulbos olfatórios leva a um aumento significativo dos

níveis de BDNF no hipocampo e córtex frontal, quando comparado ao grupo Sham. Apesar de a hipótese original indicar um déficit de BDNF na depressão, a up-regulação de BDNF hipocampal causada pela BO pode ser uma resposta adaptativa que pode ser modificada por um período de tratamento mais prolongado. Nesta mesma linha, em outro estudo foi demonstrado que ratos submetidos à BO apresentam uma diminuição da vulnerabilidade dos neurônios hipocampais à excitotoxicidade (Gary et al., 2002), sugerindo que a ablação dos bulbos estimula fatores neurotróficos hipocampais. Ressalta-se ainda que, este resultado corrobora com o aumento de pCREB hipocampal também demonstrado no presente estudo – um outro marcador em modelos animais de depressão (Chen et al., 2001). Adicionalmente, o aumento do imunoconteúdo de BDNF pode ainda ser resultado de uma up-regulação compensatória desta neurotrofina após a BO, que pôde ser prevenida pelo tratamento (14 dias) com extrato (30 mg/kg, p.o.) e com fluoxetina (10 mg/kg, p.o.).

O presente estudo também elucidou que a administração sistêmica (p.o.) do extrato de *T. avellanedae* a camundongos é capaz de produzir um efeito tipo-antidepressivo no TSC através da modulação dos receptores NMDA e da via L-arginina-óxido nítrico (NO)-guanosina monofosfato cíclico (cGMP). Além disso, este trabalho extende dados da literatura reforçando o papel da inibição dos receptores NMDA e da síntese de NO-GMPc nos mecanismos envolvidos nos efeitos dos agentes antidepressivos.

As doses sub-ativas (1 mg/kg) e ativas (30 mg/kg) de extrato de *T. avellanedae* utilizadas neste estudo foram escolhidas com base em um estudo prévio realizado pelo nosso grupo (Freitas et al., 2010). Ressalta-se ainda, que estas doses não produzem nenhum efeito sobre a atividade locomotora dos animais. É relevante mencionar que este trabalho foi realizado utilizando camundongos fêmeas, tendo em vista que a

prevalência de depressão é cerca de duas vezes maior em mulheres que em homens (Wong e Licinio, 2001).

O sistema glutamatergico desempenha um importante papel na depressão (Skolnick, 1999). O envolvimento da inibição dos receptores NMDA no mecanismo de ação dos antidepressivos convencionais e de putativos agentes antidepressivos tem sido sugerido por vários estudos que mostram que a administração de NMDA é capaz de reverter os efeitos do tipo-antidepressivo destes compostos (Brocardo et al., 2008; Nowak et al., 1993). No presente estudo, o pré-tratamento dos animais com NMDA bloqueou o efeito antidepressivo do extrato de *T. avellanedae* no TSC, sugerindo que o efeito antidepressivo do extrato também pode ser dependente da inibição da ativação dos receptores NMDA. Reforçando esta idéia, a co-administração de doses sub-ativas de extrato de *T. avellanedae* e de MK-801, um antagonista não competitivo de receptor NMDA, produziu um efeito anidepressivo sinérgico no TSC. A redução da atividade locomotora observada no grupo de animais co-tratados com extrato e MK-801 não interfere para o efeito do tipo-antidepressivo observado. Vários estudos mostram que MK-801 e outros antagonistas competitivos e não competitivs NMDA possuem ação do tipo-antidepressiva no TNF (Skolnick, 1999; Trullas e Skolnick, 1990) e TSC (Mantovani et al., 2003). Nossos resultados estão de acordo com esta linha, uma vez que compostos que reduzem a neurotransmissão através dos receptores NMDA exercem efeitos do tipo-antidepressivos (Brocardo et al., 2008; Mantovani et al., 2003; Rosa et al., 2003).

Este estudo também avaliou o envolvimento da via da L-arginina-NO-GMPc na ação do tipo-antidepressiva do extrato de *T. avellanedae*. Está bem descrito que o NO desempenha um papel importante como neuromodulador no sistema nervoso e que a

manipulação farmacológica da via NO-GMPc pode consituir uma nova abordagem terapêutica para o tratamento da depressão (Da Silva et al., 2000; Harkin et al., 1999).

No presente estudo, nós demonstramos que o pré-tratamento dos camundongos com L-arginina, substratato para a NOS, preveniu significativametente o efeito anti-imobilidade do extrato de *T. avellanedae*. De maneira semelhante, em outro estudo, o efeito antidepressivo da imipramina foi bloqueado pelo pré-tratamento com L-arginina no TNF (Harkin et al., 2004). Nossos dados sugerem que a inibição da síntese de NO pode estar contribuindo para a redução do tempo de imobilidade no TSC causada pelo extrato de *T. avellanedae*.

Reforçando a idéia de que o efeito do extrato de *T. avellanedae* no TSC seja, pelo menos em parte devido a uma inibição da síntese de NO, neste estudo demonstramos que o co-tratamento de 7-nitroindazol (um inibidor específico da síntese de NO) ou ODQ (um inibidor da GCs) com uma dose sub-ativa de extrato produziu um efeito antidepressivo sinérgico no TSC. Considerando que a NO ativa a enzima GCs, que gera GMPc, e medeia muitos dos efeitos do NO (Moncada et al., 1989), nossos resultados oferecem evidências para sugerir que o efeito do tipo-antidepressivo do extrato pode ser mediado através da redução dos níveis de GMPc, provavelmente como uma conseqüência da redução da síntese de NO.

Juntos, estes resultados estão de acordo com os achados que mostram que inibidores da NOS, dependente de suas concentrações, exercem efeitos do tipo-antidepressivos em uma variedade de modelos animais de depressão (Da Silva et al., 2000; Harkin et al., 1999; Jesse et al., 2010) e são capazes de aumentaram a atividade de antidepressivos que agem através de mecanismos serotonérgicos (Harkin et al., 2004).

Adicionalmente, outra evidência de que o extrato de *T. avellanedae* exerce seus efeitos no TSC através da diminuição dos níveis de GMPc baseia-se na reversão do

efeito anti-imobilidade do extrato de *T. avellanedae* pelo sildenafil, inibidor da enzima PDE_5. A PDE_5 catalisa a hidrólise do segundo mensageiro GMPc e é particularmente expressa no cerebelo e no hipocampo (Bender e Beavo, 2004). Portanto, estes resultados estão de acordo com o fato de que a inibição da síntese de GMPc pode ser um importante alvo os antidepressivos. A **Figura 7** ilustra um possível mecanismo de ação antidepressiva do extrato de *T. avellanedae* através do envolvimento dos receptores NMDA e a via da L-arginina-NO/GMPc.

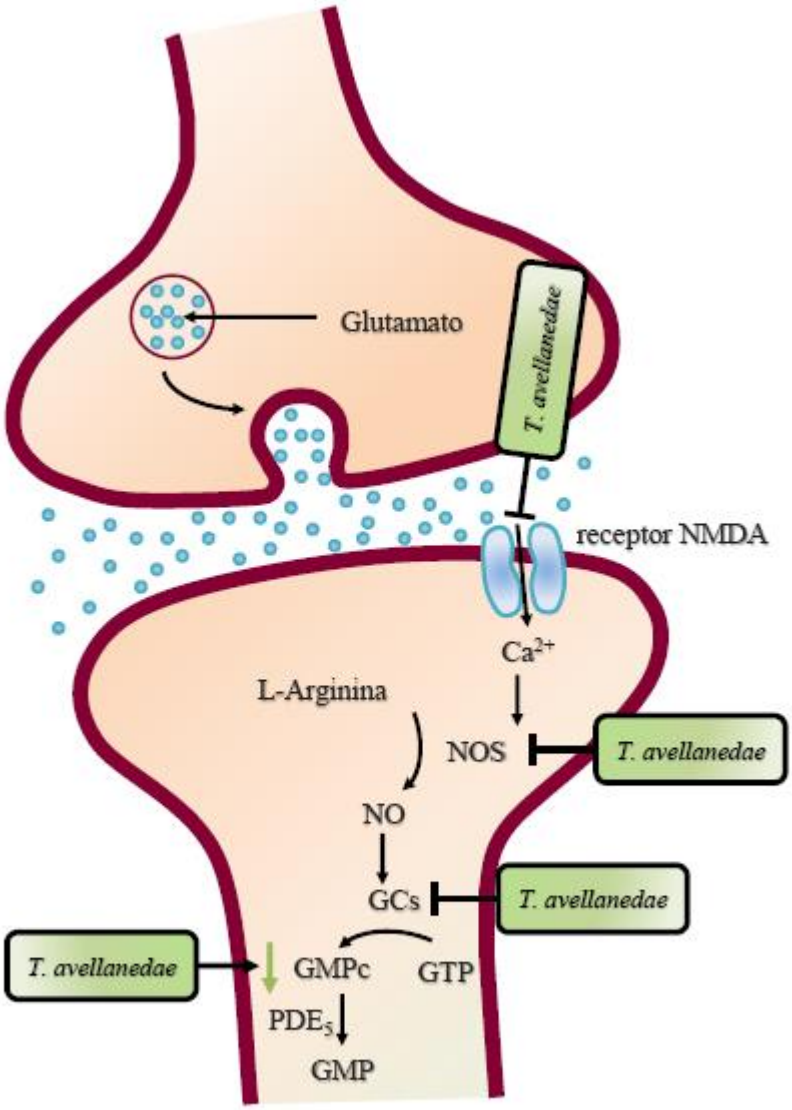


Figura 7. Envolvimento dos receptores NMDA e a via da L-arginina-NO/GMPc no efeito do tipo-antidepressivo do extrato de *Tabebuia avellanedae* no TSC. O efeito do tipo-antidepressivo do extrato de *Tabebuia avellanedae* no TSC é mediado, pelo menos em parte, pelo bloqueio de receptores NMDA, inibição das enzimas NOS e GCs e diminuição dos níveis de GMPc neuronais.

Considerando que: a) o tratamento repetido dos camundongos com extrato de *Tabebuia avellaneddae* é efetivo em prevenir sintomas depressivos no modelo de indução de depressão da bulbectomia olfatória; b) e que o tratamento agudo com extrato produz um efeito do tipo-antidepressivo no TSC dependente do bloqueio da ativação de receptores NMDA e da inibição da síntese de NO/GMPc; c) e que compostos como zinco e riluzol que também mostram-se efetivos em suprimir a neurotransmissão

glutamatérgica, e que além disso, exibem efeitos antidepressivos no modelo da bulbectomia olfatória de maneira aguda (Nowak et al., 2003; Takahashi et al., 2011), podemos supor que o extrato de *Tabebuia avellanedae* apresente uma ação antidepressiva rápida, e por este motivo, se justificaria a avaliação do seu efeito de maneira aguda no modelo da bulbectomia olfatória em estudos futuros. Vale ressaltar que um dos principais focos no estudo de novos compostos e extratos com ação antidepressiva é a busca de novos compostos dotados de ação antidepressiva mais rápida que os antidepressivos convencionais.

Em relação à caracterização fitoquímica do extrato de *Tabebuia avellanedae* por eletroferose capilar foram identificados os seguintes compostos: ácido p-hidroxibenzóico, ácido anísico, ácido verátrico e ácido caféico. Nenhum sinal característico de Lapachol ou de β-Lapachona foi observado. Estes dados corroboram com o estudo de Steinert et al. (1995) que através da técnica de HPLC (cromatografia líquida de alta performance) não encontraram nenhum sinal característico de Lapachol. Adicionalmente, Queiroz e seu grupo (2008) não identificaram a presença de β-Lapachona no extrato de *Tabebuia avellanedae* por TLC (cromatografia de camada delgada) ou HPLC. A literarura relata que o ácido p-hidroxibenzóico possui propriedades antimicrobianas (Sánchez-Maldonado et al., 2011), o ácido anísico possui ação antinflamatória (Singh et al., 2006), o ácido verátrico é anti-hipertensivo e antioxidante (Saravanakumar e Raja, 2011), e o ácido caféico é um composto antioxidante (Simić et al., 2007) que apresenta propriedades antidepressivas (Takeda et al., 2002; Takeda et al., 2003; Takeda et al., 2006; Dzitoyeva et al., 2008). Desta forma, estudos futuros com estes compostos isolados poderão elucidar sua contribuição para o efeito antidepressivo do extrato etanólico de *Tabebuia avellanedae*, mas destacamos

que com base nos dados da literatura podemos supor que o ácido caféico seja um dos principais compostos antidepressivos do extrato.

Considerando todos os resultados obtidos, podemos sugerir que o efeito do tipo-antidepressivo do extrato de *Tabebuia avellanedae* seja mediado por: estimulação da neurotransmissão noradrenérgica, serotonérgica e dopaminérgica; inibição de receptores NMDA; diminuição da síntese de NO e inibição de pGSK-3β (Ser^9) (**Figura 8**).

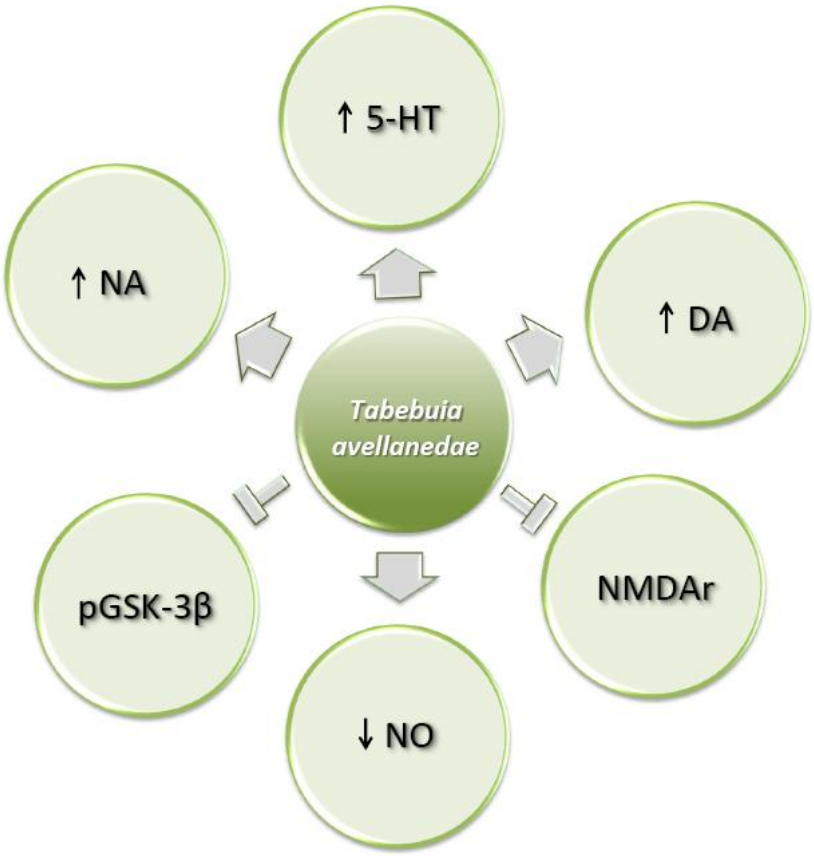


Figura 8. Possíveis mecanismos de ação envolvidos no efeito do tipo-antidepressivo do extrato de *Tabebuia avellanedae*. Podemos sugerir que seja mediado por: estimulação da neurotransmissão noradrenérgica, serotonérgica e dopaminérgica; inibição de receptores NMDA; diminuição da síntese de NO e inibição de pGSK-3β (Ser^{9}).

7. Conclusões

Em relação à avaliação do efeito antidepressivo do extrato de *Tabebuia avellanedae* e da fluoxetina em camundongos submetidos à bulbectomia olfatória (BO):

- A administração do extrato de *T. avellanedae* durante 14 dias em camundongos produziu um efeito do tipo-antidepressivo no TSC que pode estar associado com um aumento de pCREB (Ser^{133})e pGSK-3β (Ser^{9}) hipocampal;
- O modelo da BO padronizado em nosso laboratório causou hiperatividade no TCA e comportamento anedônico no splash teste, validando-o como modelo de indução de depressão;
- A ablação dos bulbos olfatórios causou um aumento significativo da via da ERK1/CREB/BDNF na região do hipocampo;
- O tratamento repetido com extrato de *T. avellanedae* foi efetivo em prevenir a hiperatividade e comportamento anedônico induzido pela BO de meneira semelhante ao antidepressivo fluoxetina;
- A ação do tipo-antidepressiva do extrato de *T. avellanedae* nos animais bulbectomizados está associada com uma redução dos níveis de fosforilação de ERK1 e do imunoconteúdo de BDNF na região do hipocampo;
- Os efeitos da fluoxetina nos animais BO são mediados por uma redução de pERK1/2, pCREB e do imunoconteúdo de BDNF hipocampal;
- O papel de cada uma dessas vias de neuroplasticidade e de neuroproteção merece mais estudos para a determinação de sua relação direta com a ação antidepressiva do extrato de *T. avellanedae* e da fluoxetina no modelo da BO.

Em relação à caracterização do mecanismo de ação antidepressiva do extrato de *Tabebuia avellanedae* através da investigação da participação do sistema glutamatérgico (via receptores NMDA) e da via da L-arginina-NO/GMPc:

- O efeito do tipo-antidepressivo do extrato de *T. avellanedae* no TSC é dependente do bloqueio da ativação de receptores NMDA e da inibição da síntese de NO/GMPc, estendendo de maneira significativa dados da literatura acerca da ação do tipo-antidepressiva desta planta.

PERSPECTIVAS

- Investigar o efeito do extrato de *Tabebuia avellanedae* administrado de maneira aguda no modelo da bulbectomia olfatória sobre alterações comportamentais e neuroquímicas induzidas por este modelo de depressão;
- Avaliar o efeito do tipo-antidepressivo dos compostos isolados e purificados encontrados no extrato de *Tabebuia avellanedae* (ácido p-hidroxibenzóico, ácido anísico, ácido verátrico e ácido caféico) no TNF, TSC e TCA e o envolvimento de vias de sinalização relacionadas à sobrevida celular neste efeito;
- Avaliar efeito antidepressivo dos compostos isolados e purificados encontrados no extrato de *Tabebuia avellanedae* (ácido p-hidroxibenzóico, ácido anísico, ácido verátrico e ácido caféico) em camundongos submetidos à BO através da reversão de alterações comportamentais e neuroquímicas induzidas por este modelo de depressão;

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